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Aberrant Expression of p14^{ARF} in Human Cancers: A New Biomarker?

Kazushi Inoue[#] and Elizabeth A. Fry

The Department of Pathology, Wake Forest University Health Sciences, Winston-Salem, NC 27157

Abstract

The *ARF* and *INK4a* genes are located on the *CDKN2a* locus, both showing tumor suppressive activity. ARF has been shown to monitor potentially harmful oncogenic signalings, making early stage cancer cells undergo senescence or programmed cell death to prevent cancer. Conversely, *INK4a* detects both aging and incipient cancer cell signals, and thus these two gene functions are different. The efficiency of detection of oncogenic signals is more efficient for the former than the latter in the mouse system. Both *ARF* and *INK4a* genes are inactivated by gene deletion, promoter methylation, frame shift, aberrant splicing although point mutations for the coding region affect only the latter. Recent studies show the splicing alterations that affect only *ARF* or both *ARF* and *INK4a* genes suggesting that *ARF* is inactivated in human tumors more frequently than what was previously thought. The *ARF* gene is activated by E2Fs and Dmp1 transcription factors while it is repressed by Bmi1, Tbx2/3, Twist1, and Pokemon nuclear proteins. It is also regulated at protein levels by Arf ubiquitin ligase named ULF, MKRN1, and Siva1. The prognostic value of ARF overexpression is controversial since it is induced in early stage cancer cells to eliminate pre-malignant cells (better prognosis); however, it may also indicate that the tumor cells have mutant p53 associated with worse prognosis. The ARF tumor suppressive protein can be used as a biomarker to detect early stage cancer cells as well as advanced stage tumors with p53 inactivation.

Keywords

ARF; INK4a; CDKN2a; DMP1 (DMTF1); p53; BMI1; E2F; expression; cancer; prognosis; biomarker

Introduction

Since the cloning of the alternate reading frame gene from the mouse *Arf/Ink4a* locus, the Arf tumor suppressor has been reported to be a sensor for hyperproliferative stimuli stemming from mutant Ras and c-Myc oncoproteins (1–3). p19^{Arf} (p14^{ARF} in humans) and p16^{Ink4a} mRNAs are generated from separate and first exons 1 β and 1 α (19.4 kilo base pairs

[#] Corresponding author: kinoue2@triad.rr.com; Phone: 336-407-1642. EAF: eafry10@hotmail.com.

Conflicts of Interest

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[kbp] apart in humans; 12.4 kbp apart in mice) which splice into two common exons 2 and 3 (Fig. 1). These two genes are different tumor suppressor since *p19^{Arf}* uses only exons 1 and 2 (also *p14^{ARF}*) while *p16^{Ink4a}* uses all of the exons 1-3 for production of the protein (4, 5). This locus has a unique genomic structure not found in other mammalian genes due to the splicing used by *Arf* which uses an alternate reading frame in the coding region of exon two. Of note this *ARF-INK4a* (*CDKN2a*) locus is located 11.5 kbp downstream of genomic loci for *CDKN2b* that encodes for p15^{INK4b} (Fig. 1). We previously reviewed aberrant transcripts from this locus (6). Since RB is regulated by p16^{INK4a} and p53 is regulated by p14^{ARF}, the *ARF/INK4a* locus is frequently inactivated in human cancers second only to p53 in frequency. Both p19^{Arf} and p16^{Ink4a} act as tumor suppressors in mice (7–9) despite a lack of amino acid sequence similarity. ARF is a highly basic, insoluble protein (pI 11) (1–3). Although human and mouse ARF differ in size (mouse 19 kDa, human 14 kDa) and show only 49% amino acid sequence identity, the functions of the ARF proteins are the same between the two species (4). The *Arf* gene exists in other mammals, such as rat, opossum, pig, hamster, and chicken (10). Ectopic Arf arrests immortal rodent cell lines such as NIH 3T3 as well as transformed human cells (5, 11), a classic phenotype of tumor suppressor genes. The main function of ARF is to quench oncogenic signals stemming from hyperproliferation, diverting it to the p53-dependent cell cycle arrest or apoptosis (1–3). The ability of Arf to inhibit cell cycle progression in a number of cell types suggested that Arf has powerful growth-inhibitory functions in cells, which stimulated researchers to study the *in vivo* activity of Arf to prevent tumors. Arf sequesters MDM2 in the nucleolus, thus preventing p53 degradation. In addition, it inhibits the transcription factor E2F activity. These activities lead to cell cycle arrest at G1 and G2 (5). Itahana et al. reported that the mitochondrial protein p32/C1QBP binds the ARF C-terminus where p32 is required for ARF to localize to mitochondria to induce apoptosis, demonstrating the essential role of ARF in tumor suppression and programmed cell death (12). Although it has been believed that most of tumor-suppressive function of Arf is mediated by p53, accumulating evidence has pointed out additional p53-independent functions of Arf through interaction with proteins such as E2Fs, c-Myc, NF-κB, HIF1α (transcription factors), nucleophosmin (NPM), and ribosomal RNAs (reviewed in 10). Recent study suggests that nuclear factor E2-related factor 2 (NRF2) is a major target of ARF in p53-independent tumor suppression (13).

p16^{INK4a} promotes cellular senescence in response to stressors, such as telomere erosion, stalled replication forks, reactive oxygen species, and oncogene activation (reviewed in 14–16). Expression of p16^{INK4a} in normal cells is almost undetectable, but once induced, p16^{INK4a} binds to cyclin-dependent kinase 4/6 (CDK4/6) to inhibit its activity, thereby promoting a retinoblastoma (RB) - dependent cell cycle arrest. This tumor suppressive mechanism limits the growth of early stage cancer cells, and accordingly, the p16^{INK4a}-CDK4/6-RB axis is disrupted in most human cancers, with inactivation of p16^{INK4a} the most common mechanism (14–16). Although the induction of p16^{INK4a} by oncogenic stimuli results in a beneficial effect to eliminate incipient cancer cells, it also accelerates cell aging (17–19). This review is focused on the mechanism of regulation of ARF and its expression in human cancers. Special interests have been put on the capacity of ARF for early stage tumor detection as well as advanced cancers with p53 mutation for potential clinical applications since it is not expressed in normal cells.

Transcriptional regulation of *Arf*

Transcriptional control is the major mechanism of *Arf* regulation since it has a long half-life of 6 hrs, much longer than those of cyclins (20, 21). A number of transcription factors regulate *Arf*, either positively or negatively (Fig. 1). The E2F1 transcription factor induces *Arf* by directly binding to its consensus sequences (22, 23) and also the Sp1 sites (23, 24) which are located in the upstream of the exon 1 β , thus activating cell growth arrest or apoptosis to avoid the emergence of incipient cancer cells (1–3). E2F2 and E2F3a also transactivate the *Arf* promoter (23). Conversely, E2F3b, a splice isoform of E2F3a that is not regulated by E2Fs, represses *Arf* transcription and stimulates cellular growth (25, 26). The observation that the loss of *Arf* can rescue E2F3b depletion-mediated cell cycle arrest suggests anti-reciprocal correlation between these two proteins (26). To prevent hyperproliferation of cells with oncogenic stress, c-Myc activates fail-safe programs such as apoptosis and cellular senescence by inducing *Arf* transcription (27). Thus, Myc-induced *Arf* activates the p53 signaling, preventing immortalization of murine embryonic fibroblasts (MEFs). Bouchard et al. reported that c-Myc signaling increases nuclear FoxO, which, in turn, binds to the *Arf* promoter to suppress c-Myc-driven lymphomagenesis (28).

Qi et al. showed that *Arf* inhibits c-Myc through physical interaction independent of p53 (29). c-Myc increases *Arf*, which in turn binds to c-Myc to block its ability to activate transcription, induce hyperproliferation, and transformation. Conversely, c-Myc's ability to repress transcription is not influenced by *Arf*, but rather c-Myc-mediated cell death is enhanced (29). This feedback represents a p53-independent mechanism to prevent c-Myc-mediated tumor development. The differential effects of *Arf* on c-Myc function indicate that independent molecular mechanisms mediate c-Myc-induced hyperproliferation and programmed cell death. The same group later showed that Egr1 mediates p53-independent c-Myc-induced apoptosis via an *Arf*-dependent mechanism (30). Therefore, c-Myc-*Arf*-binding switches the intrinsic activity of c-Myc from a proliferative to apoptotic protein independent of p53.

A cyclin D-binding Myb-like protein Dmp1 (also named Dmtf1) also transactivates the *Arf* promoter (22). Inoue et al. demonstrated that Dmp1 (Dmp1 α) binds to the Ets consensus (5'-CCCGGATGC-3') of the mouse *Arf* promoter to activate its transcription in normal MEFs, which results in *Arf*/p53-dependent cell cycle arrest (22, 31–46; Dmp1 reviewed in 6, 47–50). DMP1 α transactivates the human *ARF* promoter, the activity of which is antagonized by its splice variant DMP1 β (44, 46). E2F1 collaborates the Dmp1 to activate the *Arf* promoter to avoid neoplastic transformation of early stage cancer cells (22). Similar mechanisms must be present in human *ARF* promoter (24) since both E2F and DMP1-consensus sequences are found in the human version.

Arf is actively involved in TGF β signaling. Zheng et al. reported that TGF β increases the *Arf* mRNA levels through Smad2/3 and p38^{MAPKs} in MEFs (51). Chromatin immunoprecipitation showed that TGF β stimulates Smad2/3 binding and histone H3 acetylation 5' to exon 1 β , followed by increased *Arf* transcription and tumor suppression (51). Then the same group showed that TGF β -mediated induction of *Arf* correlated with decreased DNA-binding of C/EBP β to the *Arf* promoter (52). Their results indicate that

Smad2/3 and C/EBP β are positive and negative regulators for *Arf* transcription affected by TGF β .

Both human and mouse *ARF* promoters have binding sites for acute myeloid leukemia-1 (AML1), which causes cellular senescence in MEFs. Interestingly, the t(8;21) fusion protein AML1-ETO is frequently expressed in acute leukemia with low ARF expression (53). Consistently, Shikami et al. showed that the mRNA expression of *p14^{ARF}* in t(8;21) AML cells was found to be lower than those without t(8;21) translocation (54). Since *p14^{ARF}* has been shown to inhibit p53 degradation by binding to MDM2 (1–3), repression of *p14^{ARF}* expression in t(8;21) AML accelerates the degradation of p53 by MDM2 explaining why genotoxic damage caused by ionizing radiation does not induce p53 response in t(8;21) AML cells.

Repressors of *Arf* transcription have also been reported (47; Fig. 2). The polycomb group gene *Bmi1*-deficient MEFs shows decreased cell cycle progression and increased premature senescence, which are rescued by *Arf/Ink4a* depletion (55). *Bmi1* requires the EZH2-containing Polycomb-Repressive Complex 2 (PRC2) to repress *ARF/INK4a* transcription (56). TBX2 immortalizes MEFs and decreases senescence in normal human cells by repression of *Arf* transcription (57; Fig. 2). The basic helix-loop-helix transcription factor Twist1 activates the recruitment of EZH2 to the *Arf* transcription start site. Thus, it increases the levels of H3K27Me3 on the *ARF/INK4a* locus, followed by repression of *Arf* transcription (58). An intriguing regulator for *ARF/INK4a* is the long non-coding RNA *ANRIL* (antisense non-coding RNA in the *INK4* locus; Fig. 1), which is transcribed in the antisense orientation (56, 59, 60). *ANRIL* promotes the epigenetic repression of its tumor suppressors in the *ARF/INK4a* locus by physically interacting with the Polycomb proteins Suz12 and Cbx7, which in turn recruit the Polycomb repressive complex 1 and 2 to the locus, leading to histone modification and silencing of the locus (56).

In general, the relative importance of the *Arf* gene far exceeds that of *p16^{Ink4a}* for tumor suppression in mice reflecting the stronger tumor-prone tendency of *Arf*-deficient mice (7, 61) than *p16^{Ink4a}*-deficient mice (8, 9) leaving an open question in human cases since *in vivo* assays are not possible in the latter.

Regulation of Arf at protein levels

Although the importance of Arf in cellular senescence and tumor suppression has been well-documented, Arf is also regulated by posttranslational mechanisms. A report from the Sherr's lab suggested that Arf protein, although it lacks lysine sites, is polyubiquitinated at its N-terminus followed by proteasomal degradation by an unknown E3-ubiquitin ligase (20). Then a number of ubiquitin ligases have been isolated that regulate Arf at the protein level. In short, both transcriptional and post-transcriptional controls are important in Arf regulation.

In 2005, Chen et al. identified an ubiquitin ligase for p53, ARF-BP1/Mule, as a factor associated with *p14^{ARF}* *in vivo* (62). ARF-BP1 has a homology to the E6-AP carboxyl terminus motif, and its ubiquitin ligase activity for p53 was inhibited by ARF. ARF-BP1

directly bound and ubiquitinated p53 (62). They also reported that inactivation of endogenous ARF-BP1 was critical for ARF-mediated p53 stabilization. Thus ARF-BP1 is a crucial mediator for both the p53-dependent and p53-independent functions of the ARF tumor suppressor. In 2010, the same group reported the first E3-ubiquitin ligase for ARF named ULF (ubiquitin ligase for Arf; ULF) (63, Fig. 1). ULF caused polyubiquitination and proteasomal degradation of ARF, thus activating cell proliferation. ULF interacts with ARF both *in vitro* and *in vivo* and promoted lysine-independent ubiquitination and degradation (63). Consistently, ULF knockdown stabilized ARF in normal human cells, triggering p53-mediated growth arrest.

Chio II et al. reported that tumor necrosis factor receptor - associated death domain shuttled dynamically from the cytoplasm into the nucleus to inhibit the interaction between Arf and ULF, thereby promoting Arf protein stability and tumor suppression (64). Arf stability control is crucial for differentiating normal (low expression) versus oncogenic (high expression) levels of c-Myc, suggesting that the differential effects on ULF-mediated Arf ubiquitination by c-Myc levels act as a check point in oncogene-induced stress response (64). Very recently it was reported that the glioma tumor suppressor candidate region gene 2 (GLTSCR2) bound to ARF (65). The complexes were released to the nucleoplasm where GLTSCR2 increased the binding affinity of ARF for ULF and enhanced degradation by polyubiquitination (65). Thus GLTSCR2 is a strong candidate for promoting the subcellular localization and protein stability of ARF (65).

NPM is a multifunctional protein that forms a stable complex with ARF in the nucleolus (66). Importantly, NPM protects ARF from proteasome-mediated degradation. NPM and c-Myc, both of which are commonly overexpressed in cancer cells, promoted ARF stabilization by inhibiting ULF-mediated ubiquitination (66, 67). NPM is mutated in about one third of primary samples from AML, leading to cytoplasmic dislocation of the protein. Cytoplasmic NPM mutants found in tumor cells do not retain ARF in the nucleolus, and thus unable to stabilize the protein, compromising the activation of the ARF-p53 pathway (67). Consistently, steady levels of both Arf and p53 are very low in human AML cells expressing cytoplasmic NPM. ULF knockdown stabilized ARF and reactivated p53 responses in AML cells, suggesting that ULF is a *bone fide* E3 ligase for ARF. They even suggested a novel therapeutic for AML by inhibiting ULF (67).

Ko and colleagues reported the second E3-ubiquitin ligase, Makorin 1 (MKRN1), which targets Arf (68; Fig. 1). *MKRN1*-deficient MEFs showed decreased cell growth with simultaneous increase in Arf protein levels (68). Consistent with these findings, MKRN1 was shown to induce ubiquitination and proteasomal degradation of Arf (69).

Siva1, the third E3-ubiquitin ligase for ARF, induces the proteasomal degradation of ARF thus inhibiting p53 function (70; Fig. 1). Through direct interaction, Siva1 promoted the ubiquitination and degradation of ARF, which in turn, affected the stability of p53. Functionally, Siva1 regulated cell cycle progression and cell proliferation in an ARF/p53-dependent manner (70). Together, these findings suggest that the mechanisms of ARF regulation at the protein level are critically important in its responses to oncogenic stress.

Decreased expression of ARF in human cancer

Consistent with the findings in gene knock-out mice, point mutation, promoter methylation, and/or deletion of the *ARF/INK4a* locus in human cancers have been reported (21, 71, 72; Table 1). However, it is difficult to evaluate the relative importance of p16^{INK4a} and p14^{ARF} individually since mutations or deletions at the *ARF/INK4a* locus frequently affects both proteins. Generally, exon 2 is the site of point mutation, affecting mainly p16^{INK4a}. Point mutations that cause gene frameshift affect both genes. Some of these exon 2 mutations change ARF localization and affect its regulation of downstream target proteins (73, 74). Silencing of the *ARF* gene promoter through hypermethylation is frequently observed in human cancers (75) although the frequency was 8 times lower in *ARF* than *INK4a* in non-small cell lung carcinomas (37). Simultaneous methylation of both *ARF* and *INK4a* is a common occurrence in samples from the accelerated phase of chronic myeloid leukemia (76).

Although it has been shown that p16^{INK4a} is the major player in human tumor suppression, accumulating pieces of information suggest that p14^{ARF} plays a significant role in tumor suppression. In one study, loss of p14^{ARF} expression was found in 38/50 glioblastomas, with 29 showing either homozygous deletion or hypermethylation (75); *ARF* was specifically deleted in nine of the samples. An exon 1β mutation that altered the growth-inhibitory properties and intracellular localization of human p14^{ARF} was reported in a melanoma (75). A germline deletion of exon 1β has been reported in patients for a family predisposed to malignant melanoma with/without *INK4a* involvement (77, 78). Splicing mutations that affect both *ARF* and *INK4a* have been reported (79–81); a splicing error that affects *ARF*, but not *INK4a* has also been reported (82). More recently, the *ARF-INK4a* hybrid transcript (chARF) has been reported in a melanoma cell line: the fusion protein has ARF activity, but not that of *INK4a* (83). *ARF* haploinsufficiency due to a germline mutation in exon 1β was observed in a family of three individuals with melanoma or breast cancer (84), which had been proved in a mouse model (8).

ARF overexpression in human cancer

Silva et al. (85) analyzed ARF inactivation mechanisms with 100 primary breast carcinomas. RT-PCR showed altered expression of the *p14^{ARF}* transcript, with 17% overexpression and 26% decreased expression. No detectable alterations were found in cases with overexpression, but 77% of tumors with decreased expression for *p14^{ARF}* showed one or more genetic alterations (86; Table). A correlation was found between decreased *p14^{ARF}* expression and poor prognostic parameters. They reported that promoter hypermethylation was present in 31% for *16^{INK4a}* and 50% for *p14^{ARF}* in tumors with decreased expression. In conclusion, these tumor suppressors are often co-altered in human breast cancer (86).

The prognostic value of p14^{ARF} expression was studied in HPV16(+) squamous cell carcinoma of the oropharynx (SCCOP) (87). They determined the HPV16 status and expression of p14^{ARF} and p53 in 552 patients. Patients having rs3731217 TG/GG variant genotype for *ARF* polymorphism (high ARF) were two to three times more likely to have HPV16(+) compared with tumors with homozygous TT genotype (low ARF). Thus p14^{ARF}

expression predicts HPV16(+) SCCOP tumor patients with longer survival associated with better response to therapy (87).

Both of BMI1 and EZH2 are epigenetic modifiers of the polycomb group. However, EZH2 overexpression correlates with a poor prognosis in breast cancer while BMI1 overexpression correlates with a good outcome (Figure 2). Pietersen et al. (88) found that *INK4a/ARF* was expressed in those with EZH2 overexpression, but not with BMI1. They found a significantly higher proportion of p53 mutations in tumors with high EZH2, which will explain why those with high EZH2 respond poorly to therapy. In conclusion, the prognostic value for ARF should be determined simultaneously with the p53 status because ARF is overexpressed in p53 mutant tumors.

Future directions

We have reviewed *ARF* alterations in cancer. *ARF* is inactivated in human cancer by gene deletion, frame shift, promoter methylation, and point mutations that affect gene splicing. The prognostic significance for *ARF* inactivation in cancer is controversial: LOH for the *ARF/INK4a* locus does not affect the survival of breast cancer patients (43). Although it is possible that tumors with ARF expression have better prognosis due to accelerated removal of incipient cancer cells, overexpression of ARF is also an indicator for p53 mutation, which is associated with increased tumor invasion/metastasis, increased angiogenesis, and resistance to chemo/radio therapy (89). The prognosis of such patients is poor. Prognostic studies that simultaneously quantitate 14^{ARF} and p53 expression are lacking; it is thus extremely important to conduct clinical studies by quantitating ARF and p53 levels in the same sample. p14^{ARF} can be used as a biomarker to detect cancer cells since it is not expressed in non-transformed cells.

Recent studies suggest that ARF-induced, p53-independent tumor suppression is significantly abrogated by NRF2 overexpression (13). Thus NRF2 is a major target of p53-independent tumor suppression by ARF; it also suggests that the ARF-NRF2 interaction acts as a new checkpoint for oxidative stress responses. Extensive studies should be done in this field since p53 is mutated in half of human cancers.

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The *INK4b/ARF/INK4a* locus

Figure 1.

The structure for the human *p15^{INK4b}-p14^{ARF}-p16^{INK4a}* locus. The genomic structure is well-conserved between human and mice, and thus gene knockout studies have been extensively conducted in mice. The distance between exon 1 β and exon 1 α is 19.4 kbp in humans and 12.4 kbp in mice. The exon 1 α is 3.8 kbp upstream of exon 2 in humans; 5.2 kbp in mice (from 5' of exon 1 α to 5' of exon 2). The *ARF-INK4a* (*CDKN2a*) locus is located 11.5 kbp apart from the genomic locus for *CDKN2b* that encodes for p15^{INK4b} in humans (from 3' of exon 2 for *p15^{INK4b}* to 5' of exon 1 β). All of *p15^{INK4b}*, *p19^{Arf}*, and *p16^{INK4a}* genes act as tumor suppressors as reported by Krimpenfort et al. (8, 90). The DMP1 consensus is located -2.3 kb and -0.31 kb of *ARF* (shown in red reverse triangles) and -4.04 kb and -1.40 kb of *INK4a* (pink reverse triangles) in humans. Both of these are Dmp1 target genes although the mode of regulation is different (40).

Pasmant et al. identified a new large antisense noncoding RNA (named *ANRIL*; 59, 60) to this genomic locus, with a first exon located in the promoter of the *p14^{ARF}* gene and

overlapping the two exons for *p15^{CDKN2b}*. Expression of *ANRIL* was simultaneously found with p14^{ARF} both in physiologic and pathologic conditions. Kobayashi et al. found that that p14^{ARF} regulates the stability of the p16^{INK4a} protein in human and mouse cells (91). Importantly, ARF promoted rapid degradation of p16^{INK4a} protein, which was mediated by the proteasome and, more specifically, by interaction of ARF with one of its subunits, regenerating islet-derived protein 3 γ . Thus there is a significant crosstalk between ARF and INK4a at the protein level (91). ULF, MKRN1, and Siva1 are E3 ligases for ARF that accelerates its degradation (63, 64, 68–70).

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Figure 2. Oncogenic and tumor suppressive signaling involving Dmp1 and Arf.

Positive input signals for Arf have been shown in red (our own research) or pink (research from other labs). Conversely, negative signals have been shown in black. The output signals for Arf, Mdm2, and p53 are shown in striped arrows. Arf is induced by potentially oncogenic signals stemming from overexpression of oncogenes such as c-Myc, E2F1, and activated Ras, which quenches inappropriate mitogenic signaling by diverting incipient cancer cells to undergo p53-dependent growth arrest or cell death. Positive input signals for Arf have been shown in red arrows. Conversely, negative signals for Arf have been shown in black. The output signals for Arf, Mdm2, and p53 are shown in striped arrows.

Dmp1 α transactivates the *Arf* promoter in response to oncogenic stresses, and physically interacts with p53 to neutralize all activities of Mdm2 to activate the p53 pathway in response to DNA damage (1–3). Both *Dmp1*^{-/-} and *Dmp1*^{+/-} mice show hypersensitivity to develop tumors in response to carcinogen or γ -irradiation (32, 33). D-type cyclins inhibit Dmp1's activity in a Cdk-independent fashion in promoters lacking E2F sites; however, it cooperates with Dmp1 α to activate the *Ink4a* and *Arf* promoters to eliminate incipient tumor cells (31, 40). The *Dmp1* promoter is activated by the oncogenic Ras-Raf-Mek-Erk-Jun and HER2-Pi3k-Akt-NF- κ B pathways, and thus Ras or HER2-driven carcinogenesis is accelerated in *Dmp1*-deficient mice (34, 37, 39). The human *DMP1* locus generates three splice variants, namely *DMP1* α , β , and γ with antagonizing activity between DMP1 α and β (46). *DMP1* β and γ transcripts have not been reported in mice. Dmp1 α physically interacts with the epigenetic modifier YY1 that affects EZH2 activity. YY1 binds to HDM2 and Dmp1 α to accelerate HDM2-mediated polyubiquitination of p53. Our study shows that Dmp1 α physically interacts with p53 through p53's carboxyl-terminal and Dmp1's DNA-binding domain (42). Dmp1 α antagonized p53's ubiquitination by HDM2 both *in vitro* and *in cell* and restored p53's nuclear localization that had been lost with HDM2 expression (42); Dmp1 also stabilized p53 binding to transcriptional target genes (45). Dmp1 α -p53 interaction increases the levels of p53 independent of Dmp1's DNA-binding, and hence both

p21^{Cip1} and *Bbc3* promoters were synergistically activated by co-expression of Dmp1 α and p53 in *p53*^{-/-}; *Arf*^{-/-} cells (42). In accordance, the induction of *p21^{Cip1}* and *Bbc3* by genotoxic drug treatment was more seriously affected in *Dmp1*^{-/-} and *p53*^{-/-} tissues than in *Arf*^{-/-} (42). In summary, Dmp1 α stimulates the p53 pathway by direct transactivation of the *Arf* promoter in response to oncogenic stresses (34, 37, 39, 40) and direct physical interaction with p53 in DNA damage response (DDR; 42, 45).

Mekk1 is activated by a variety of oxidative stress signaling, such as dsDNA breaks, UV, cytokines, osmotic stress, and oncogenes. Activation of MEKK1 by c-Abl in DDR has been reported. MEKK1 is cleaved by caspase 3 following DNA damage to generate MEKK1, which increases the Dmp1 α protein by phosphorylation. Loss of *PTEN* is found in 70 % of advanced prostate cancer (PCa), resulting in activation of the Pi3k-Akt pathway that promotes survival by inhibiting apoptosis and causing genomic instability. The tumor suppressor *Pten* accelerates the conversion of Pip3 to Pip2, and thus is a negative regulator of Pi3k signaling pathway. In PCa, loss of *PTEN* drives cell cycle arrest and senescence as a tumor suppressive mechanism mediated by upregulation of p53 expression. Accumulating studies show that RNA splicing is affected by DDR, and also roles of YY1 and PTEN in DDR. There are feed forward/back regulations between p53 and Mdm2, p53 and Arf, and p53 and Dmp1. In addition, Arf negatively regulates Mdm2, and Mdm2 negatively regulates p53 as published.

Table 1.
Mechanisms of gene inactivation of the *ARF-INK4a* locus

Gene	Mechanism of inactivation	Human cancers affected	Impact on prognosis	References
ARF	gene deletion/insertion			1–3, 71, 72, 75, 77, 78
	promoter hypermethylation			37, 75, 76, 85, 86
	frame shift			
	splicing errors			79–82
		liver		
		lung (LOH 30%, methylation 7%)		37
		kidney		
		brain tumor (glioblastoma, astrocytoma, etc)		
		chronic myelogenous leukemia		76
		breast (LOH 20%)	LOH does not have impact	43, 85, 86
ARF	overexpression	malignant melanoma		
		HPV(+) head & neck tumor	better prognosis	87
		breast, 17%		85, 86

The Arf tumor suppressor gene is either underexpressed or overexpressed in human cancers, which will cause dysfunction. ARF overexpression can reflect the p53 inactivation of the tumor, suggesting the role for detection of tumor cells with p53 mutation.