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# Epigenetic regulation of the *INK4b-ARF-INK4a* locus

## In sickness and in health

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**The *INK4b-ARF-INK4a* locus encodes for two cyclin-dependent kinase inhibitors, p15$^{INK4b}$ and p16$^{INK4a}$, and a regulator of the p53 pathway, ARF. In addition ANRIL, a non-coding RNA, is also transcribed from the locus. ARF, p15$^{INK4b}$ and p16$^{INK4a}$ are well-established tumor suppressors which function is frequently disabled in human cancers. Recent studies showed that single nucleotide polymorphisms mapping in the vicinity of ANRIL are linked to a wide spectrum of conditions, including cardiovascular disease, ischemic stroke, type 2 diabetes, frailty and Alzheimer disease. The *INK4b-ARF-INK4a* locus is regulated by Polycomb repressive complexes (PRCs) and its expression can be invoked by activating signals. Other epigenetic modifiers such as the histone demethylases JMJD3 and JHDM1B, the SWI/SNF chromatin remodeling complex and DNA methyltransferases regulate the locus interplaying with PRCs. In view of the intimate involvement of the *INK4b-ARF-INK4a* locus on disease, to understand its regulation is the first step for manipulate it to therapeutic benefit.**

**Key words:** senescence, p16$^{INK4a}$, ARF, p15$^{INK4b}$, ANRIL, polycomb, histone demethylases, DNA methylation

**Abbreviations:** ANRIL, antisense non-coding RNA at *INK4a/ARF* locus; ARF, alternative reading frame; GWAS, genome-wide association studies; *INK4a*, *INK4b*, inhibitors of CDK4 a and b; JMJD3, jumonji-domain containing 3; LD, linkage disequilibrium; OIS, oncogene-induced senescence; PcG, polycomb group; PRC, polycomb group repressive complexes; SNP, single nucleotide polymorphism

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### Introduction

The *INK4b-ARF-INK4a* locus spans around 35 kb on human chromosome 9p21 that contains the *INK4b (*also termed *CDKN2b)*, *ARF* and *INK4a* genes (these two jointly referred as *CDKN2a*, reviewed in ref. 1 and **Fig. 1**). They encode for two cyclin dependent kinase inhibitors, p15$^{INK4b}$ and p16$^{INK4a}$ and an unrelated protein, p14$^{ARF}$. Whereas p15$^{INK4b}$ is translated from its own independent genetic structure, ARF and p16$^{INK4a}$ share their second and third exons. However, ARF splices exon 2 in an alternative reading frame to that of p16$^{INK4a}$, hence the name ARF.[2] *ARF* and *INK4a* are transcribed from independent promoters. Both p15$^{INK4b}$ and p16$^{INK4a}$ bind specifically to CDK4 and CDK6 [3] blocking cell proliferation by preventing phosphorylation of RB resulting in a $G_1$ arrest. ARF sequesters MDM2 in the nucleolus.[4] This in turn activates p53 resulting in either cell cycle arrest or apoptosis.[3] Recently, a new large antisense non-coding RNA termed ANRIL (also known as *CDKN2b* antisense or *CDKN2BAS*) has been mapped to the *INK4b-ARF-INK4a* locus[5] (**Fig. 1**) where it is presumed to play a regulatory role. How ANRIL and other non-coding RNAs regulate the expression of the locus is currently the matter of active investigation.

### The *INK4b-ARF-INK4a* Locus and Disease

The interest on the *INK4b-ARF-INK4a* locus originated from genetic linkage studies showing the association of mutations or deletions on chromosome 9p21 with familial predisposition to melanoma.[6,7] It was subsequently demonstrated that in addition to germ-line mutations, homozygous deletion on 9p21 is one of the most frequent cytogenetic events associated with a wide variety of tumors (reviewed in ref. 8). Loss of the *INK4b-ARF-INK4a* locus is the most frequent copy number alteration across tumors and cancer cell lines.[9,10] Multiple studies have revealed p16$^{INK4a}$ as the main tumor suppressor

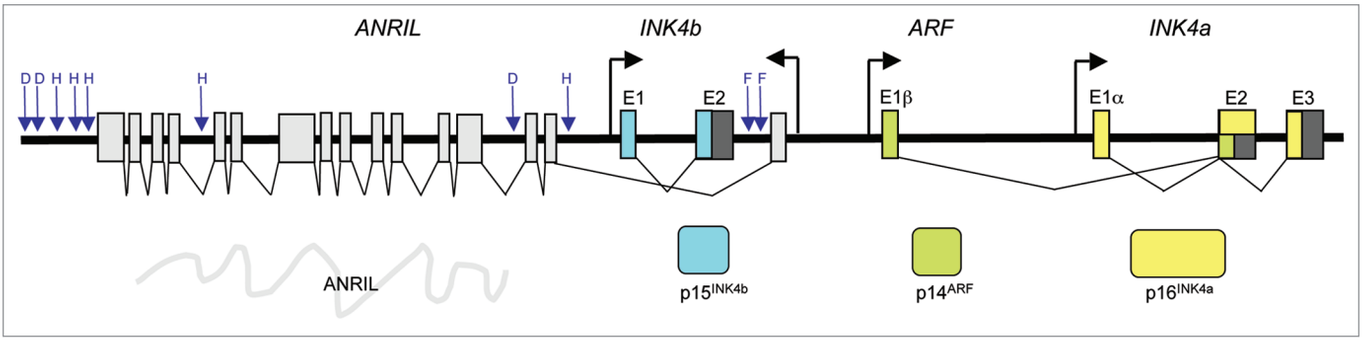


**Figure 1.** Organization of the *INK4b-ARF-INK4a* locus and disease-associated SNPs. Genetic structure of the human *INK4b-ARF-INK4a* locus. The coding exons are shown in colors and non-coding exons are shown in light gray for ANRIL and dark gray for the other genes of the locus. The approximate position of single nucleotide polymorphisms (SNPs) associated with disease states is indicated by blue arrows. SNPs associated with type 2 diabetes mellitus (D), vascular heart disease (H) and frailty (F) are indicated. Map is not drawn to scale and positions are approximate.

in the locus while showing that $p15^{INK4b}$ and $p14^{ARF}$ can also act as tumor suppressors. Intragenic mutations that inactivate *INK4b* or *ARF* are observed, though rare in comparison to those affecting *INK4a*. For example, specific inactivation affecting *ARF* but not *INK4a* can occur in melanoma,[11] while methylation of the *INK4b* promoter is observed in hematopoietic malignancies.[12] Mouse models have confirmed that deficiency for either of the proteins encoded by the *INK4b-ARF-INK4a* locus, alone or in combination results in tumor-prone animals.[8,13] It is worthy to mention that despite mouse models have been clearly useful to dissect the involvement of the *INK4b-ARF-INK4a* locus in health and disease, significant differences exist in its regulation between mouse and human. Most notably while mouse $p19^{Arf}$ is upregulated during replicative or Ras-induced senescence, human $p14^{ARF}$ is not (reviewed in ref. 1).

An explanation for the frequent alteration of the locus in cancer is its activation in response to aberrant oncogenic signalling. As such, members of the *INK4b-ARF-INK4a* locus are key effectors of oncogene-induced senescence (OIS) and are induced in premalignant lesions, limiting tumor progression. Therefore, to progress to a more malignant state, a lesion suffers insurmountable pressure to silence the locus through deletion, mutations or epigenetic regulation. The *INK4b-ARF-INK4a* locus is also upregulated at replicative senescence and aging.[8] In murine tissues, increased expression of $p16^{Ink4a}$ and $p19^{Arf}$, but not of $p15^{Ink4b}$, is observed with aging,[14,15] making the case for an involvement of the *INK4b-ARF-INK4a* locus in age-related pathologies. Again, the difference in the locus regulation between mouse and human should be taken into account and although $p16^{INK4a}$ expression increases with aging in humans, there are no reports of a similar increase for $p14^{ARF}$ levels.[16] Additional evidence for an extended role of the *INK4b-ARF-INK4a* locus in disease came from a series of linkage studies in which single nucleotide polymorphisms (SNPs) in a region spanning 120 kb around the *INK4b-ARF-INK4a* locus were associated with increased susceptibility to frailty,[17] coronary artery disease,[18,19] myocardial infarction,[20] type 2 diabetes[21-23] and late onset Alzheimer disease.[24] Interestingly different SNPs have been associated with increased disease risk on those studies (**Fig. 1**), suggesting that not a single polymorphism is responsible for the increased susceptibilities observed.

## Regulation of the *INK4b-ARF-INK4a* Locus by Polycomb Repressive Complexes

Given the extraordinary relevance of the *INK4b-ARF-INK4a* locus on disease, it is key to maintain it repressed under normal circumstances but without losing the ability to induce its expression when needed. A critical layer to achieve this control is epigenetic regulation through Polycomb (Pc) repressive complexes (PRC1 and 2). The PRC2 complex establishes the repressive H3K27met3 chromatin mark, catalyzed by the histone methyltransferase activity of EZH2.[25-27] This epigenetic mark, is recognized by the PRC1 maintenance complex, which in addition monoubiquitinates histone H2A.[28] The key role of PRCs in regulating the *INK4b-ARF-INK4a* locus is remarked by the fact that overexpression of different PcG members such as Bmi1, Cbx7 or Cbx8 results in repression of the locus and bypass or delays senescence.[29-31] Conversely, cells lacking PRC1 components such as Bmi1 or Ring1b[29,32] show aberrant expression of the *INK4b-ARF-INK4a* locus. Mice knockout for different PcG genes have multiple developmental problems. In particular $Bmi1^{-/-}$ mice have skeletal transformations and severe neurological and hematopoietic defects.[33] With the exception of the skeletal alterations that are due to deregulation of the Hox gene cluster, the rest of the defects observed are restored to a great extent by knocking out *Ink4a/Arf*.[34]

## Non-coding RNAs: ANRIL and Others

A remaining question is how PRCs are targeted to the *INK4b-ARF-INK4a* locus. The core members of the PRCs do not have a DNA binding motif and the assumption is that they must associate through accessory factors to target DNA. In Drosophila, PREs (polycomb recruiting elements) have been identified, and some transcription factors such as Pho named as responsible of the recruitment of PRCs to those elements. Pho is required for PcG-mediated silencing in Drosophila.[35] Interestingly, the homolog of Pho, the transcription factor YY1,[36] is

also involved in PcG-mediated silencing in mammals.[37] However, despite studies showing genome-wide PcG distribution in mammalian chromatin, equivalent PRE and targeting factor(s) has not yet been clearly identified for mammalian systems.[38] It has been proposed that a combination of association with transcription factors and long interfering non coding RNAs could be responsible of the recruitment of PRC to their target genes in mammalian cells.[38] Recently, a DNA element recognized by YY1 has been shown to target PRC complexes to the HoxD locus.[39] However, a similar arrangement in the *INK4b-ARF-INK4a* locus has not been identified so far. On the other hand, evidence is starting to show that long non-coding RNAs such as ANRIL contribute to the targeting of PRCs to the *INK4b-ARF-INK4a* locus. LincRNAs can control gene expression through tethering chromatin-modifying complexes to specific genomic loci. Multiple lincRNAs interact with PRC2 and other chromatin modifier complexes.[40] In addition, a novel class of short RNAs transcribed from the 5' end of Polycomb target genes interact with PRC2 and could play a role in PRC association to target genes.[41] Chromobox proteins, such as those that are part of the PRC1, cannot only bind methylated histones but also to RNA. For example, treatment with RNAse decreases the association of CBX7 with H3K27me3 and the inactive X chromosome.[42] Treatment with RNAse or mutation of residues needed for CBX7 to interact with RNA also results in reduced recruitment of CBX7 to the *INK4b-ARF-INK4a* locus with the corresponding effects on senescence.[43] LincRNAs are currently seen as a platform helping in the recruitment of different chromatin remodeling complexes, such as PRC2 and LSD1/CoREST/REST.[40] Although ANRIL seems to control the levels of $p15^{INK4b}$ acting as an antisense transcript,[44] ANRIL and probably other non coding RNAs (long and short) can also regulate the *INK4b-ARF-INK4a* locus by contributing to the recruitment of epigenetic factors. Single nucleotide polymorphisms (SNPs) in a 58 kb-long interval on chromosome 9p21 have previously been associated with an increased susceptibility to coronary heart disease.[18,20] ANRIL maps to this same region (**Fig. 1**).[5,45] A relation between disease-associated SNP close to ANRIL and the expression of the members of the *INK4b-ARF-INK4a* locus has been noted in human samples.[46,47] Recently, a deletion of the orthologous 70 kb-long non-coding intervals on mouse chromosome 4 was also found to affect the cardiac expression of the neighboring $p15^{Ink4b}$ and $p16^{Ink4a}$ genes.[48] Whether ANRIL or cis-regulatory regions are mediating this effect needs to be investigated.

## Activation of the *INK4b-ARF-INK4a* Locus by Oncogenic Signals

To fulfill its function as a stress sensor, the *INK4b-ARF-INK4a* locus must be kept repressed in basal conditions, but also be quickly activated when needed. A number of transcription factors have been linked with the activation of the locus as a whole or their individual members and this have been reviewed in reference 1, In particular, we have a better knowledge of the transcription factors involved in activating $p16^{INK4a}$ in response to oncogenic stress such as Ras expression, where the relation between Ets2 factors and Id1 is key.[49]

Parallel to activate or mobilize transcription factors to induce the *INK4b-ARF-INK4a* locus, stimuli that trigger its induction have to modify the epigenetic status of the locus and wipe their repressive marks. To achieve this during replicative and oncogene-induced senescence there is a change in the expression and recruitment of the key enzymes regulating the methylation of H3K27. Levels of EZH2, the enzyme that methylates H3K27 decrease during replicative senescence and OIS.[50-52] In parallel, the H3K27 demethylase JMJD3 is upregulated in response to Ras, recruited to the *INK4b-ARF-INK4a* locus and regulates the activation of the locus by oncogenic stress. JMJD3 is found upregulated in some preneoplastic lesions as nevi and have credentials to be a tumor suppressor, as it is frequently deleted in different tumor types.[51,52] How JMJD3 is regulated by Ras is a matter of active investigation. Another mechanism mediating the activation of the *INK4b-ARF-INK4a* locus is the direct modification and displacement of polycomb proteins. MAPKAP, which is activated downstream of Ras can phosphorylate Bmi1 prompting its release from chromatin.[53] However, whether this phosphorylation-mediated displacement occurs during senescence has not been investigated.

## Chromatin Remodeling by the SWI/SNF Complex

Additional chromatin modifiers and epigenetic marks also control the status of the locus, sometimes in direct interplay with PRCs. The upregulation of genes normally repressed by PRCs need of chromatin remodeling complexes, such as the SWI/SNF (or BAF) complex.[54] The gene encoding *SNF5* (also termed SMARCB1 or BAF47), one of the components of the SWI/SNF complex is frequently deleted in malignant rhabdoid tumors (MRT). By analyzing MRT tumors it was noted that *SNF5* is needed to regulate $p16^{INK4a}$ expression through recruitment of the SWI/SNF complex and Polycomb eviction and the deletion of *SNF5* results in reduced expression of $p16^{INK4a}$.[55,56] There is a crosstalk between SWI/SNF-mediated chromatin remodeling and the PRC complexes (**Fig. 2**). An interesting question is whether the SWI/SNF complex plays an active role in activating the *INK4b-ARF-INK4a* locus in response to oncogenic stress or in specific tissues. Recent evidence seems to suggest so, as Snf5 acts as a prominent mediator of $p19^{Arf}$ expression in murine sarcomas generated upon activation of K-Ras. It has been suggested that this can account for the differences in susceptibility to transformation by Ras of different tissues.[57]

## Regulation of the *INK4b-ARF-INK4a* Locus by DNA Methylation

In addition to homozygous deletion and inactivating mutations, one of the most common mechanisms of inactivation of *INK4a* in cancer is through aberrant promoter methylation.[58] Similarly, DNA promoter methylation inactivating *INK4b* is observed in a number of hematological malignancies.[12] It is becoming evident that DNA methylation occurs not in isolation but in close relation with other

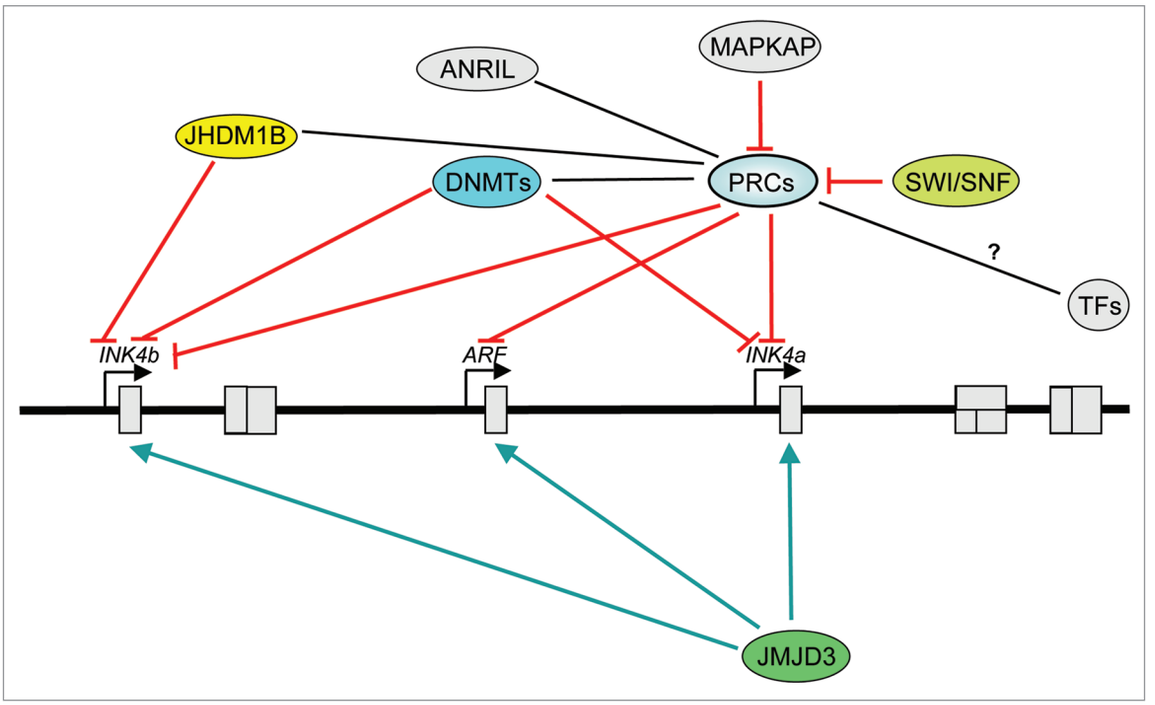


**Figure 2.** Epigenetic regulation of the *INK4b-ARF-INK4a* locus. Cartoon summarizing different epigenetic mechanisms regulating the locus. Epigenetic silencing of the locus in normal cells is mediated by Polycomb repressive complexes (PRCs). In tumorigenesis, methylation of the *INK4a* or *INK4b* promoters is often observed. Chromatin remodeling by the SWI/SNF5 complex result in displacement of PRCs complexes and locus activation. The histone demethylases Jhdm1b regulate the expression of $p15^{INK4b}$ while JMJD3 counteract the effects of H3K27me3 marks and PRC-mediated silencing. MAPKAP phosphorylation of Bmi1 results in PRC1 displacement from chromatin. Recent evidence suggests that ANRIL and maybe other ncRNAs could regulate the locus. We still do not understand how transcription factors interplay with this epigenetic machinery to regulate the locus. Epigenetic modifiers are shown in color. PRCs, Polycomb repressive complexes; DNMTs, DNA methyl transferases; TFs, transcription factors. Black lines show relation; red arrows, activation; green arrows, inhibition. Map is not drawn to scale and positions are approximate.

epigenetic modifications such as PRC-mediated silencing. This link is highlighted by the fact that members of the PRC1 and PRC2 complexes, such as EZH2 and CBX7, can interact physically with the DNA methylation machinery, binding to Dnmt3b.[59,60] In addition, Polycomb target genes are more often represented among those aberrantly methylated in cancer,[61-63] adding functional consequences to these physical interactions. Further highlighting the relation between the different epigenetic remodelers of the *INK4b-ARF-INK4a* locus, restoration of an active SWI/SNF complex by reintroduction of SNF5 in deficient cells results not only in the eviction of PRC complexes from the *INK4b-ARF-INK4a* locus but in a loss of DNMT3b localization and DNA methylation in the locus.[56]

## Additional Chromatin Modifiers Controlling the *INK4b-ARF-INK4a* Locus

Recently another histone demethylase, Jhdm1b/Kdm2b, has also been linked with regulation of *INK4b-ARF-INK4a* locus. Initial interest on this enzyme arose from insertional mutagenesis studies that showed its association with tumorigenesis, although there was controversy as if a tumor suppressor or as an oncogene.[64,65] It was first suggested that Jhdm1b/Kdm2b demethylates H3 lysine 4 (H3K4me3)[66] but currently the strongest biochemical evidence in vitro and in vivo suggests that Jhdm1b controls demethylation of H3 lysine 36 (H3K36me3).[67] Ectopic expression of Jhdm1b/Kdm2b bypasses replicative senescence.[64] Independent studies suggest that regulation of the *Ink4b-Arf-Ink4a* locus mediates its effects on senescence.[68] More specifically it has been proposed that Jhdm1b/Kdm2b controls $p15^{Ink4b}$ expression.[67] How modification of H3K36, a mark normally present in recently transcribed genes to prevent reinitiation at intragenic sites can regulate the expression of $p15^{Ink4b}$ is not clear.[69] A possible explanation can be found in the observation that Jhdm1b/Kdm2b can form part of complexes containing PcG members,[70] suggesting a link between this chromatin remodeling enzyme and PRC (**Fig. 2**).

## Concluding Remarks

Recent GWAS have highlighted that besides being key actors on tumor suppression the members of the *INK4b-ARF-INK4a* locus may play important roles on other diseases. At the moment multiple laboratories are trying to better understand what the relevant SNPs located around the *INK4b-ARF-INK4a* locus mean for its expression and how they affect its regulation. In particular the question of whether these SNPs are affecting the levels or function of the non-coding

RNA ANRIL or maybe unveil the presence of cis-regulatory elements needs to be answered. The interplay between different chromatin modifiers with PRCs on its centre is complex (**Fig. 2**). We will need also to better understand how cellular signals such as oncogenic stress are integrated by transcription factors and the epigenetic machinery to regulate the *INK4b-ARF-INK4a* locus. The *INK4b-ARF-INK4a* locus has been analyzed on cancer from a diagnostic and prognostic perspective, as very often mutations or deletions make its alterations irreversible. However in a subset of tumors, epigenetic modifications, reversible by nature, contribute to silence the locus. If a functional *INK4b-ARF-INK4a* locus is also present in other diseases and it is still open to regulation, we could envision mechanisms and drugs that could contribute to restore its normal function. Clever mice models have shown that there is potential to control the locus obtaining beneficial effects (i.e., cancer protection) while averting unwanted side effects (i.e., accelerated aging).[71] To fully understand how the *INK4b-ARF-INK4a* locus is regulated in normal and pathological circumstances is the essential first step for its therapeutic manipulation.


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