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Review

Transcriptional Regulation of the *p16* Tumor Suppressor Gene

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Abstract. *The p16 tumor suppressor gene encodes a specific inhibitor of cyclin-dependent kinase (CDK) 4 and 6 and is found altered in a wide range of human cancers. p16 plays a pivotal role in tumor suppressor networks through inducing cellular senescence that acts as a barrier to cellular transformation by oncogenic signals. p16 protein is relatively stable and its expression is primary regulated by transcriptional control. Polycomb group (PcG) proteins associate with the p16 locus in a long non-coding RNA, ANRIL-dependent manner, leading to repression of p16 transcription. YB1, a transcription factor, also represses the p16 transcription through direct association with its promoter region. Conversely, the transcription factors Ets1/2 and histone H3K4 methyltransferase MLL1 directly bind to the p16 locus and mediate p16 induction during replicative and premature senescence. In the present review, we discuss the molecular mechanisms by which these factors regulate p16 transcription.*

Cell fate determinants, such as differentiation, cell growth, senescence and apoptosis, are mediated through regulation of the G_1 phase of the cell cycle. Progression of G_1 in mammalian cells is controlled by two classes of cyclin and cyclin-dependent kinase (CDK) complexes: cyclin Ds-CDK4/6 and cyclin Es-CDK2 (1, 2). The kinase complexes inactivate the retinoblastoma protein (pRB) family *via* phosphorylation leading to pRB-E2F dissociation and promoting progression to S phase (3). CDK inhibitors (CKIs), including p15, p16, p18, p19, p21, p27 and p57, specifically bind to and inhibit the activity of cyclin-CDK complexes, thus preventing G_1-to-S progression (4, 5). Among these CKIs, p16 plays a pivotal role in the regulation of cellular senescence through inhibition of CDK4/6 activity (6, 7). Cellular senescence acts as a barrier to oncogenic transformation induced by oncogenic signals, such as activating *RAS* mutations, and is achieved by accumulation of p16 (Figure 1) (8-10). The loss of p16 function is, therefore, thought to lead to carcinogenesis. Indeed, many studies have shown that the *p16* gene is frequently mutated or silenced in various human cancers (11-14).

Although many studies have led to a deeper understanding over the biochemical and cellular functions of p16, the regulation of p16 expression is still poorly understood. We (15, 16) and Bracken *et al*. (17) have reported that polycomb group (PcG) proteins bind to and silence the *INK4* locus encoding *p15, p16* and *ARF via* histone H3 lysine27 (H3K27) trimethylation. A long non-coding RNA (lncRNA), *ANRIL*, is required for the PcG proteins complex recruitment on the *INK4* locus (18, 19). Recently, we also reported that Y box binding protein 1 (YB1) directly binds to and represses *p16* transcription resulting in the prevention of cellular senescence (20). In contract, Ets1 and 2 transcription factors (21) and MLL1 histone methyltransferase/CUL4-DDB1 ubiquitin ligase complexes (22) bind to and activate *p16* transcription during replicative and premature senescence. In the present review, we will focus on PcG proteins, *ANRIL*, YB-1, Ets1/2 and the MLL1/CUL4-DDB1 complex and discuss the molecular mechanisms by which they regulate *p16* transcription.

Repression of *p16* Transcription by PcG Proteins

PcG proteins form multimeric protein complexes, polycomb repression complex (PRC)-1 and -2, that stably repress target gene expression. EZH2, a catalytic component of PRC2, methylates histone H3K27 of the target locus, which recruits PRC1 to the region. PRC1 then ubiquitinates histone H2AK119 leading to repression of target gene transcription

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(23-25). We and Bracken *et al*. revealed that both PRC1 and PRC2 are involved in the repression of the *INK4* locus encoding *p15* and *p16* (and Arf in the mouse) (15-17). Forced expression of BMI1, a component of PRC1, decreases the levels of *p16* and *p15* mRNA leading to an increase in cell proliferation. In contrast, depletion of BMI1, EZH2 or SUZ12, a component of PRC2, results in increased expression of p15 and p16 mRNA, thus causing cellular senescence. Chromatin immuneprecipitation (ChIP) showed that both PRC1 and PRC2 associate with the *INK4* locus. During premature and replicative senescence, PRC1 and PRC2 dissociate from *INK4* leading to abrogation of histone H3K27 tri-methylation resulting in the increase of *p16* expression. Taken together, these data provide a model in which PcG proteins bind to and repress the *p16* transcription though histone H3K27 tri-methylation. Barradas *et al*. and Agger *et al*. showed that histone H3K27 demethylase JMJD3 mediates the activation of *p16* (and *Arf* in mouse) by oncogenic RAS (26, 27) supporting the notion that *p16* is epigenetically repressed *via* PRC2-mediated histone H3K27 trimethylation (Figure 2).

The Recruitment of PcG Proteins on *p16* Locus by ANRIL

Recent studies have revealed that many lncRNAs are involved in cell fate determination, such as cancer development, apoptosis and differentiation (28-30). Among them, several lncRNAs have been reported to have a direct role in the recruitment of PcG proteins on target locus leading to repressing gene expression (31-35). *ANRIL* is a lncRNA, which is transcribed from between promoters of *p15* and *ARF* in the opposite transcriptional direction to these genes. We (18) and Yap *et al*. (19) reported that *ANRIL* is involved in the recruitment of PcG proteins to the *INK4* locus. Inhibition of *ANRIL* increases *p15* and *p16* transcription causing the inhibition of cell proliferation and induction of cellular senescence. A ChIP assay showed that inhibition of *ANRIL* disrupts the binding of PRC1 and PRC2 proteins on *INK4* locus. RNA immunoprecipitation (18) and an *in vitro* binding assays (19) showed that *ANRIL* associates with both PRC1 and PRC2 proteins. Collectively, these data provide a model in which *ANRIL* associates with and recruits PRC1 and PRC2 to the *INK4* locus leading to the repression of *p15* and *p16* transcription (Figure 2).

Recently, we reported that the level of *ANRIL* expression is decreased by exogenous and endogenous expression of oncogenic *RAS* (36). It has been shown that *p16* is induced by oncogenic *RAS* causing premature senescence to protect cells from hyperproliferation (8-10). The decrease of *ANRIL* expression might be required for *p16* activation and induction of premature senescence.

Activation of p16 Transcription by MLL1 and CUL4-DDB1 Complexes

We showed that MLL1 histone H3K4 methyltransferase and CUL4-DDB1 ubiquitin ligase complexes are involved in the activation of *p16* transcription by oncogenic *RAS* (22). MLL1 is a Trithorax group (TrxG) protein, which has histone H3K4 methyltransferase activity (37). In the transcriptional regulation of *HOX* genes, polycomb proteins and TrxG proteins act in an opposing manner. MLL1 associates and forms complex with RbBP5, Ash2L and WDR5, which are required for H3K4 methyltransferase activity (38, 39). Depletion of MLL1 or RbBP5 reduces the level of *p16*. Chromatin immunoprecipitation has shown that MLL1 associates with the *p16* locus (22). Interestingly, MLL1 binds to *p16* not only in the cells in which *p16* transcription is activated but also in the cells in which *p16* transcription is repressed by PcG proteins. The latter observation suggests that PcG protein-mediated repression of *p16* transcription acts dominantly over MLL1-mediated activation of *p16* transcription.

It has been shown that CUL4-DDB1-ROC1 E3 ubiquitin ligase binds to WD40 proteins, including RbBP5 and WDR5, which are required for the histone H3K4 methyltransferase activity of MLL1 (40-42). Depletion of CUL4 or DDB1 results in a decrease of histone H3K4 methylation (41). We have shown that silencing DDB1 decreases the abundance of histone H3K4 tri-methylation at the *p16* locus resulting in a decrease of *p16* expression (22), thus suggesting that the CUL4-DDB1 complex is required for MLL1-mediated activation of *p16* transcription. ChIP showed that CUL4A binds to *p16*, as well as MLL1. Silencing *MLL1* or *DDB1* abolishes oncogenic *RAS*-induced *p16* activation. Taken together, these results suggest that MLL1 and CUL4A-DDB1 complexes bind to and activate *p16* transcription in response to oncogenic *RAS* (Figure 2). However, the biochemical mechanisms by which CUL4A-DDB1 ubiquitin ligase affects MLL-mediated *p16* activation remain unclear.

Regulation of p16 by Transcription Factors Ets1/2 and YB1

Ohtani *et al*. showed that the transcription factors Ets1 and Ets2 directly bind to and activate the promoter of *p16* during replicative and premature senescence (21). Over-expressing Ets2 in human diploid fibroblasts increases *p16* expression causing cellular senescence. Id1 binds to and inhibits Ets2 leading to the repression of *p16* transcription. During replicative senescence, the level of Id1 expression is reduced but, in contract, the level of Ets1 is increased. These data support a model in which Id1 represses *p16* transcription *via* inhibiting Ets1/2 in young cells but, following reduction of Id1, Ets1/2 activates *p16* transcription causing cellular senescence.

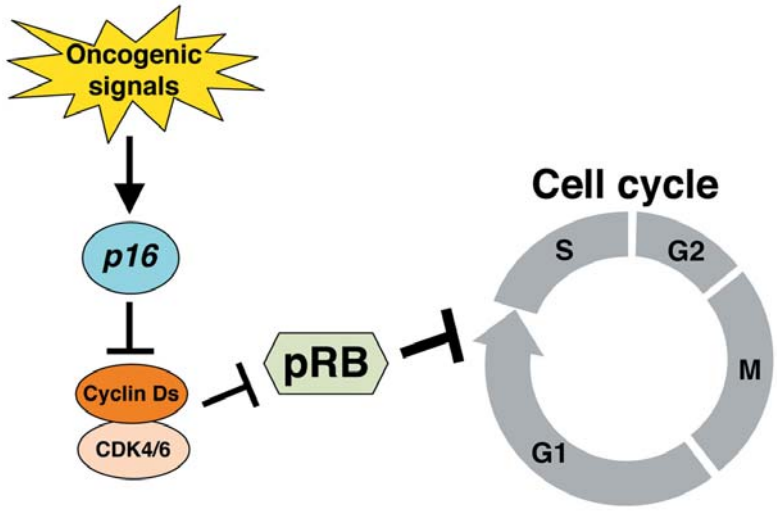

Figure 1. *Involvement of p16 in pRB-mediated cell cycle arrest induced by oncogenic signals. p16 is induced by oncogenic signals, resulting in pRB-mediated G1 arrest to protect cells from hyperproliferation.*

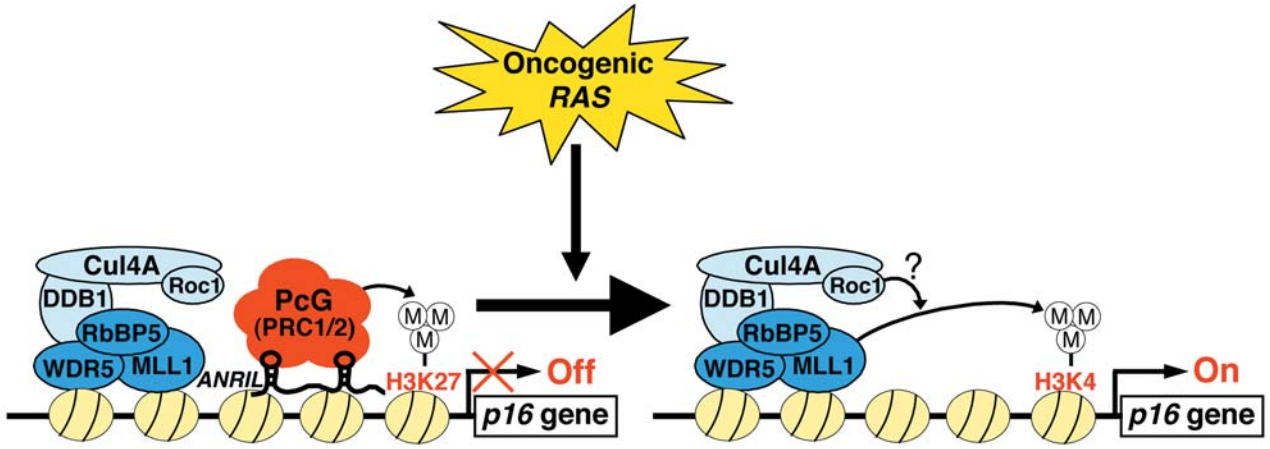

Figure 2. *The model for regulation of p16 transcription by PcG proteins and MLL1/CUL4A-DDB1 complexes. In young and unstressed cells, PcG proteins bind to and repress p16 transcription though histone H3K27 trimethylation. Oncogenic RAS signal dissociates PcG proteins from p16 locus resulting in the activation of p16 transcription through MLL1/CUL4A-DDB1-mediated histone H3K4 trimethylation.*

Recently, we reported that a transcription factor YB1 is involved in the repression of p16 (20). YB1 expression is reduced during replicative and premature senescence and the reduction in YB1 levels is associated with an increase in *p16* expression. Silencing YB1 increases *p16* mRNA levels. In contract, forced expression of YB1 in primary mouse embryonic fibroblasts decreases *p16* expression and increases the rate of cell proliferation resulting in a decrease in the number of senescent cells. ChIP showed that YB1 associates with the p16 promoter. Collectively, these data suggest that YB1 binds to and represses *p16* transcription leading to the promotion of cell proliferation and prevention of cellular senescence. However, the biochemical mechanisms underlying the function of Ets1/2 and YB1 in *p16* regulation remain unclear. It will be interesting to investigate the functional relationship between Ets1/2 and MLL1/CUL4A-DDB1 complexes in *p16* activation or YB1 and PcG proteins/*ANRIL* in *p16* repression.

Conclusion

Extensive studies over the past twenty years have revealed the biochemical and physiological function of *p16* in tumor suppression. Loss or silencing of *p16* are observed in a wide range of human cancers and is thought to be a requisite step for tumorigenesis. It is, therefore, possible that the regulators of *p16*, such as PcG proteins, *ANRIL*, YB1, MLL1, CUL4A-DDB1 and Ets1/2, are involved in tumorigenesis. Indeed, high expression of PcG proteins is observed in several human cancers and this corresponds to a decrease in p16 expression (43-45). A single-nucleotide polymorphism in the *ANRIL* locus is associated with plexiform neurofibromas in patients with neurofibromatosis type 1 (46). Over-expression of YB1 is related to tumor aggression in various human cancers (47). Therefore, the disruption of *p16* regulation by these factors may lead to aberrant cell proliferation leading to malignant transformation.

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