

DDB1-CUL4 and MLL1 Mediate Oncogene-Induced *p16^{INK4a}* Activation

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Abstract

The induction of cellular senescence by oncogenic signals acts as a barrier to cellular transformation and is attained, in part, by the elevation of the *p16^{INK4a}* tumor suppressor gene. *p16* expression is repressed epigenetically by Polycomb, but how *p16* is induced is not known. We report here that the *p16* locus is H3K4-methylated in highly expressing cells. H3K4 methyltransferase MLL1 directly binds to and is required, along with its core component RbBP5, for the induction of *p16* by oncogenic Ras. We further show that damaged DNA binding protein DDB1 and CUL4, which assemble distinct E3 ubiquitin ligases by recruiting various WD40 proteins, act upstream of MLL1-mediated H3K4 methylation. We showed that CUL4A directly binds to *p16* and that silencing DDB1 blocks Ras-induced *p16* activation. Ras expression dissociates BMI1 from the *p16* locus, whereas both CUL4 and MLL1 bind to the *p16* locus similarly in both normal and oncogenic stimulated cells. These results suggest that DDB1-CUL4 and MLL1 complexes constitute a novel pathway that mediates *p16* activation during oncogenic checkpoint response and is repressed by the polycomb repression complexes during normal growth of young cells. [Cancer Res 2009;69(5):1809–14]

Introduction

p16^{INK4a} encodes a specific inhibitor of the D-type cyclin-dependent kinases (CDK) Cdk4 and Cdk6 (CDK4/6) and is frequently mutated or inactivated in a wide range of human cancers (1, 2). Mice deficient for *p16* are more prone to spontaneous cancers (3, 4) and have a reduced decline of stem and progenitor cell function during aging (5–7). In contrast to its well-established biochemical property as a CDK inhibitor and physiologic function in tumor suppression and cell aging, little is known about the molecular mechanism regulating the expression level of *p16*. INK4 proteins (*p16^{INK4a}*, *p15^{INK4b}*, *p18^{INK4c}*, and *p19^{INK4d}*) are relatively stable and the primary regulation of INK4 is through transcriptional control.

The expression of individual *INK4* genes is distinctly regulated during development, in various adult tissues, and in response to different cellular conditions (1, 2). The expression of *p16* is undetectable during embryogenesis and in young tissues (8, 9), due in large part to the transcriptional repression by the collaborative function of Polycomb repression complex (PRC) and RB family proteins (10–14). The expression of *p16* increases markedly with

aging in many tissues and contributes to an irreversible growth arrest, termed replicative senescence, in both differentiated and stem and progenitor cells during aging (5–7). Recently, hyperproliferative stimulation resulting from ectopic oncogene expression has been shown to cause similar cellular senescence, which is accompanied by the induction of *p16* (15, 16). The molecular mechanism underlying oncogene-induced *p16* activation is not known and is investigated in this study.

Materials and Methods

Antibodies and immunologic procedures. Antibodies to BMI1 (F6; Upstate), *p16* (Ab-4; Neomarkers), MLL1 (BETHYL Laboratories, Inc.), RbBP5 (BETHYL Laboratories, Inc.), WDR5 (ab22512; abcam), H-Ras (OP23; Calbiochem), 3m-H3K4 (ab8580; abcam), 3m-H3K27 (Upstate), tubulin (DM1A; Neomarkers), and actin (C-11; Santa Cruz) were purchased commercially. Polyclonal antibodies to CUL4A (MADEAPRKGFSF-SALVGRNG) and DDB1 (REKEFNKGPWKQENVE) were raised by immunizing rabbits with synthetic peptides conjugated to keyhole limpet hemocyanin. Rabbit polyclonal antibody to MLL1-C was kindly provided by Dr. Y. Dou (University of Michigan, Ann Arbor, MI).

Cell culture, transfection, and retroviral transduction. The early passage normal human diploid fetal lung fibroblast WI38 cells were purchased from American Type Culture Collection. WI38 cells were cultured in MEM supplemented with 10% fetal bovine serum (FBS), and 293T cells was cultured in DMEM supplemented with 10% FBS. Cell transfection was performed using calcium phosphate buffer. WI38 cells were infected with a retrovirus expressing human papillomavirus oncoprotein E7 that binds to and functionally inactivate Rb family proteins. Retroviruses encoding shRNAs silencing *MLL1*, *WDR5*, *RbBP5*, *Menin*, and control *GFP* were constructed by ligating respective oligonucleotides (*MLL1*, GAAGTCA-GAGTGC GAAGTC; *WDR5*, CACCTGTGAAGCCAAACTA; *RbBP5*, GAGCC-GAGATGGTCATAAAA; *Menin*, GTCGCAAGTGCAGATGAAG; *GFP*, GCTACGTCCAGGAGCGCAC) into a pSuper-retro vector. pBabe H-Ras^{G12V} was kindly provided by Channing Der (University of North Carolina at Chapel Hill, Chapel Hill, NC). Retrovirus expressing shRNA silencing DDB1 has been described previously (17). The retroviral production and transduction were performed as previous described (13).

Quantitative reverse transcription-PCR. Total RNA was extracted by RNeasy (Qiagen), and 1 μ g was used for cDNA synthesis primed with Oligo(dT)₂₀ primers (Invitrogen). The cDNA was added to a quantitative reverse transcription-PCR mixture that contained 1 \times SYBR Green PCR master mix (Applied Biosystems) and 500 nmol/L gene-specific primers. Assays were performed in triplicate on a 7900 HT sequence detection system (Applied Biosystems). The PCR protocol comprised incubations at 50°C for 2 min and 95°C for 10 min, followed by 40 cycles, each consisting of 95°C for 15 s and 60°C for 1 min. The expression level of each gene was normalized with glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Specific PCR pairs used in this study have been described (13).

Chromatin immunoprecipitation assay. Chromatin immunoprecipitation (ChIP) assays were performed as previous described (13). Briefly, 5 \times 10⁶ WI38 cells were treated with 1% formaldehyde for 10 min. The cross-linking was stopped by the addition of 0.125 mol/L glycine and incubated for 5 min. The cells were lysed with cell lysis buffer on ice

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[10 mmol/L Hepes (pH 7.9), 0.5% NP40, 1.5 mmol/L MgCl₂, 10 mmol/L KCl, 0.5 mmol/L DTT and protease inhibitor cocktail]. After centrifugation at 4,000 rpm for 5 min, the cell pellets were lysed by sonication on ice with nuclear lysis buffer [20 mmol/L Hepes (pH 7.9), 25% glycerol, 0.5% NP40, 0.42 mol/L NaCl, 1.5 mmol/L MgCl₂, 0.2 mmol/L EDTA, and protease inhibitor cocktail]. After centrifugation at 13,000 rpm for 10 min in a cold room, the lysates were diluted with equal volume of dilution buffer [1% Triton X-100, 2 mmol/L EDTA, 50 mmol/L NaCl, 20 mmol/L Tris-HCl (pH7.9), and protease inhibitor cocktail]. Specific PCR pairs used in this study have been described (13).

Results

Transcriptional activation of *p16* is associated with H3K4 trimethylation. We (13) and others (14) recently found that the *p16* locus is histone H3 lysine27 (H3K27)-trimethylated and repressed by Polycomb group (PcG) proteins. pRB proteins are required for the recruitment of PcG proteins to *p16* locus (13). We found that associated with the decrease of PRC-mediated H3K27 methylation and activation of the *p16* locus in WI38/E7 cells in which pRB family proteins are functionally inactivated (Fig. 1A and C), H3K4 trimethylation, a prevalent marker associated with transcriptional activation, was increased broadly in the genome (Fig. 1B) and specifically in the *p16* locus (Fig. 1C and D). These data indicate that the *p16* locus is epigenetically regulated through

both repressive and active trimethylation at H3K27 and H3K4, respectively.

MLL1 and RbBP5 are required for the *p16* activation. The findings that an increase of H3K4 trimethylation at the *p16* locus is associated with its transcriptional activation and that PcG and Trithorax group (TrxG) proteins act in an opposing manner in the regulation of *Hox* genes led us to test whether MLL1, a TrxG gene encoding a SET domain H3K4 methyltransferase, is involved in *p16* activation. We found that knocking down MLL1 in WI38 cells substantially reduced both p16 protein and *p16* mRNA (Fig. 2A). The level of p18^{INK4c} mRNA, but not two other *INK4* genes (p15^{INK4b}, p19^{INK4d}), were also decreased. This result is consistent with previous findings that the *p18* locus is bound and transcriptionally activated by a MLL1 complex (18, 19) and also indicates that *INK4* genes are transcriptionally regulated differently. ChIP assay showed that MLL1 directly binds to the *p16* locus (Fig. 2B). Notably, MLL1 binds to *p16* not only in WI38/E7 cells where BMI1 is dissociated from *p16*, but also in WI38/mock cells where *p16* is bound and repressed by BMI1, indicating that BMI1-mediated *p16* repression is dominant over MLL1-mediated *p16* activation and that BMI1 antagonizes MLL1 by a mechanism independent of competition for the same binding site.

A common feature of several H3K4 methyltransferase complexes, including MLL1, is the presence of a trimeric complex consisting of three core components, RbBP5, Ash2L, and WDR5, which are

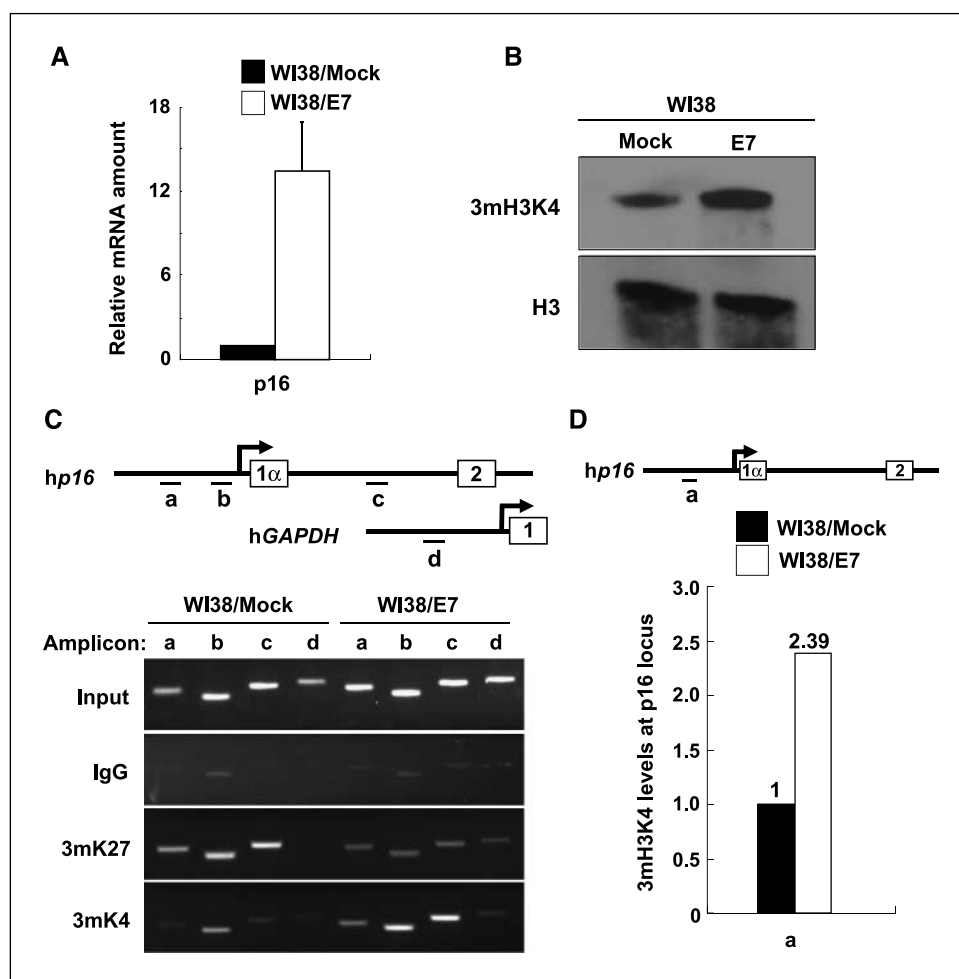
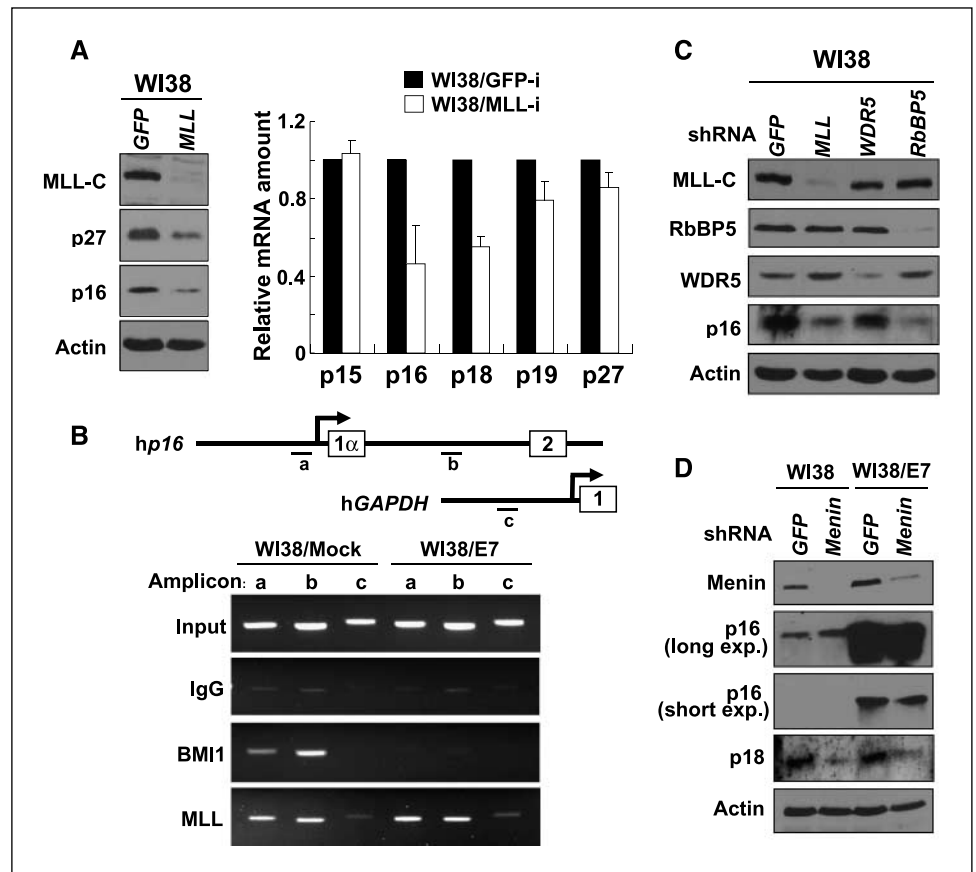


Figure 1. Transcriptional activation of *p16* is associated with H3K4 trimethylation. **A**, the levels of *p16* mRNA were determined in WI38/Mock and WI38/E7 cells by quantitative real-time reverse transcriptase PCR (Q-RT-PCR). Data are expressed relative to the corresponding values for WI38/Mock cells; *columns*, mean values were calculated from triplicates of a representative experiment; *bars*, SD. **B**, immunoblotting of H3K4 trimethylation levels of histones extracted from WI38/Mock and WI38/E7 cells with an antibody specific for trimethylated H3K4. Total histone H3 was confirmed as a loading control. **C**, a schematic representation of the human *p16* and *GAPDH* gene loci and amplicons (*a*, *b*, *c*, and *d*) used for ChIP assays. Antibodies against trimethyl-H3K27, trimethyl-H3K4, and IgG control were used in the ChIP assay. PCR was carried out using primers for each amplicon. **D**, abundance of trimethyl-H3K4 on the *p16* locus was determined by Q-PCR following ChIP using trimethyl-H3K4 antibody in WI38/Mock and WI38/E7 cells.

Figure 2. *MLL1* and *RbBP5* are required for the *p16* activation. **A**, WI38 cells were infected with a retrovirus vector encoding shRNA against either *GFP* or *MLL1*, selected by puromycin treatment for 3 d and harvested 8 d after initial infection. The efficiency of *MLL1* silencing and the effect of *MLL1* silencing on *p27* and *p16* protein expression were determined by immunoblotting. The effects of *MLL1* silencing on the expression of the four *INK4* and *p27* genes were determined by Q-RT-PCR. **B**, a schematic representation of the human *p16* and *GAPDH* gene loci and amplicons (*a*, *b*, and *c*) used for ChIP assays. Antibodies against *BMI1*, *MLL1*, and IgG control were used in the ChIP assay. PCR was carried out using primers for each amplicon. **C**, WI38 cells were infected with a retrovirus vector encoding shRNA silencing individually *GFP*, *MLL1*, *WDR5*, or *RbBP5* genes, selected by puromycin treatment for 3 d and harvested 8 d after initial infection. The efficiency of silencing each gene and the effect of silencing on *p16* expression were determined by immunoblotting. **D**, WI38 and WI38/E7 cells were infected with a retrovirus vector encoding shRNA against either *GFP* or *Menin*, selected by puromycin treatment for 3 d and harvested 8 d after initial infection. The efficiency of silencing *Menin* and the effect of silencing on *p16* and *p18* expression were determined by immunoblotting.



required for H3K4 methyltransferase activity (20, 21). Knocking down *RbBP5* reduced the level of *p16* (Fig. 2C), further supporting a direct function of *MLL1* in *p16* activation. Knocking down *WDR5* resulted in only a slight decrease of *p16*, probably due to

incomplete silencing. Several groups reported that menin associates with *MLL1* and menin-dependent H3K4 methylation maintains the expression of various genes, including multiple *HOX* genes (22, 23) and CDK inhibitors *p27* and *p18* (18, 19). This

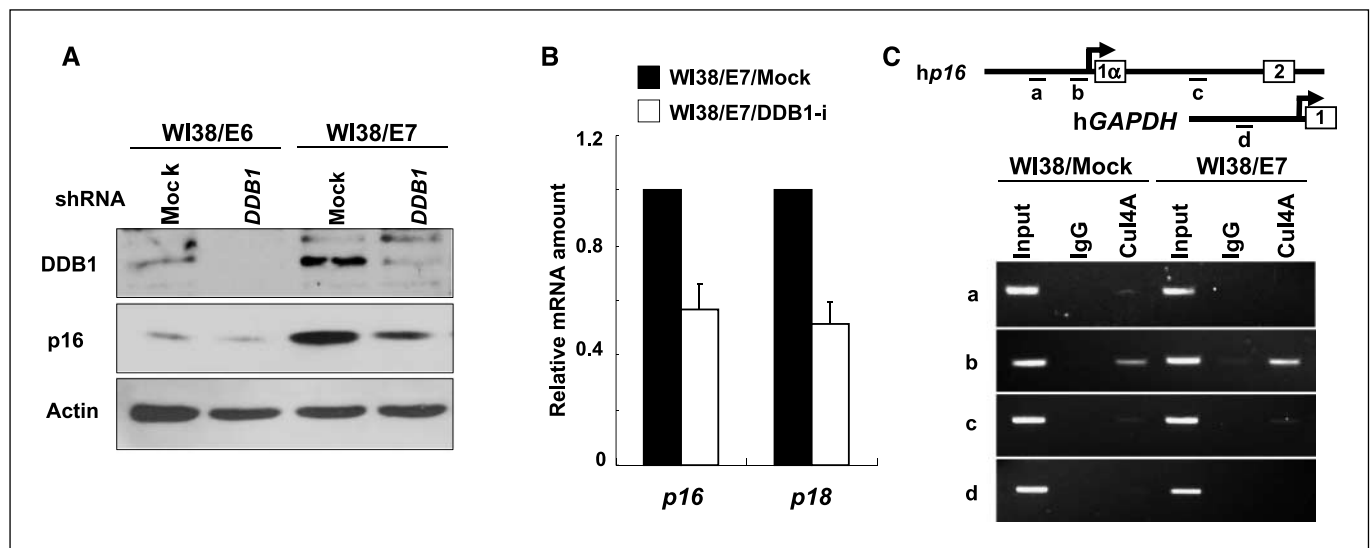


Figure 3. *CUL4A-DDB1* binds to and is required for *p16* expression. **A**, WI38/E6 and WI38/E7 cells were infected with an empty retrovirus vector (*Mock*) or retrovirus vector encoding shRNA against *DDB1*, selected by puromycin treatment for 3 d and harvested 8 d after initial infection. The efficiency of *DDB1* silencing and the effect of *DDB1* silencing on *p16* expression were determined by immunoblotting. **B**, the effect of *DDB1* silencing on the expression of *p16* was determined by Q-RT-PCR. **C**, a schematic representation of the human *p16* and *GAPDH* gene loci and amplicons (*a*, *b*, *c*, and *d*) used for ChIP assays. Antibodies against *Cul4A* and IgG control were used in the ChIP assay. PCR was carried out using primers for each amplicon.

led us to test whether menin is involved in *p16* activation. Knocking down Menin decreased *p18* expression, but not *p16* expression (Fig. 2D), indicating that menin is not involved in *p16* activation and that these two *INK4* genes are regulated differently by MLL. Supporting this, genome-wide analysis of Menin binding revealed that Menin and MLL1 localize to the promoters of thousands of human genes but do not always bind together (24), suggesting the presence of multiple distinct MLL1 complexes.

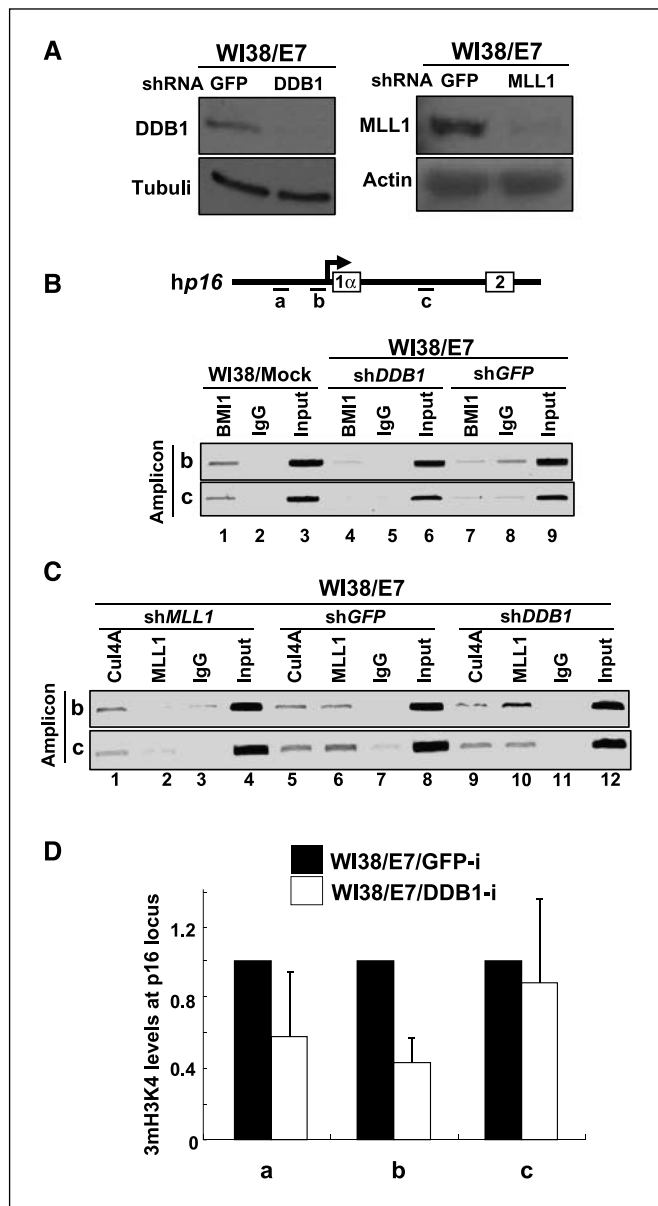


Figure 4. Silencing DDB1 reduces H3K4 trimethylation of *p16* promoter. **A**, WI38/E7 cells were infected with retrovirus vectors encoding shRNA against *GFP*, *MLL1*, or *DDB1*, and selected by puromycin treatment for 3 d and harvested 8 d after initial infection. Silencing of DDB1 or MLL1 was determined by immunoblotting. **B**, antibodies against BMI1 and IgG control were used in the ChIP assay to examine the binding of BMI1 to *p16* promoter. PCR was carried out using primers for each amplicon as indicated. **C**, antibodies against MLL1, CUL4A, and IgG control were used in the ChIP assay. PCR was carried out using primers for each amplicon as indicated in **B**. **D**, abundance of trimethyl-H3K4 on the *p16* locus was determined by Q-PCR following ChIP using with trimethyl-H3K4 antibody in WI38/E7 cells with siRNA targeting either *GFP* or *DDB1*. PCR was carried out using primers for each amplicon as indicated in **B**.

CUL4A-DDB1 binds to and is required for *p16* expression.

We (17) and others (25–27) recently discovered that DDB1, a 127 kDa protein initially identified as a component of the UV-damaged DNA-binding protein, binds to several WD40 proteins containing a DDB1 binding motif, including both RbBP5 and WDR5, and bridges them to the CUL4-ROC1 catalytic core to assemble into various distinct WD40-DDB1-CUL4-ROC1 E3 ubiquitin ligases. Both RbBP5 and WDR5 are required for MLL1 H3K4 methyltransferase activity (20, 21). Moreover, silencing CUL4 or DDB1 causes a decrease of H3 methylation, especially H3K4 methylation (25). These findings led us to test whether DDB1-CUL4 is involved in *p16* activation. We found that knocking down DDB1 reduced *p16* (and *p18*) not only in WI38/E7 cells, but also in WI38/E6 cells where, although p53 is functionally inactivated, the RB family proteins remain functional and PRC is effective in repressing *p16* (Fig. 3A and B). This result indicates that DDB1 is required not only for the marked increase in expression seen in association with RB pathway inactivation (as in WI38/E7 cells) but also the more physiologic increase in *p16*^{INK4a} expression that occurs stochastically with serial passage of WI38 cells to induce their senescence. ChIP assay showed that CUL4A binds to the *p16* locus (Fig. 3C). A direct binding of DDB1 to *p16* promoter is yet to be experimentally shown due to the lack of a DDB1 antibody capable of precipitating DDB1. As in the case of MLL1-*p16* binding, CUL4A bound to the *p16* locus in *p16*-repressed WI38/Mock nearly as well as in *p16*-activated WI38/E7 cells, suggesting again that PRC-mediated repression dominantly inhibits DDB1/CUL4A-mediated activation of the *p16* locus and that competing for the binding site(s) in the *p16* locus is not the major mechanism for either PRC-mediated repression or CUL4-mediated activation.

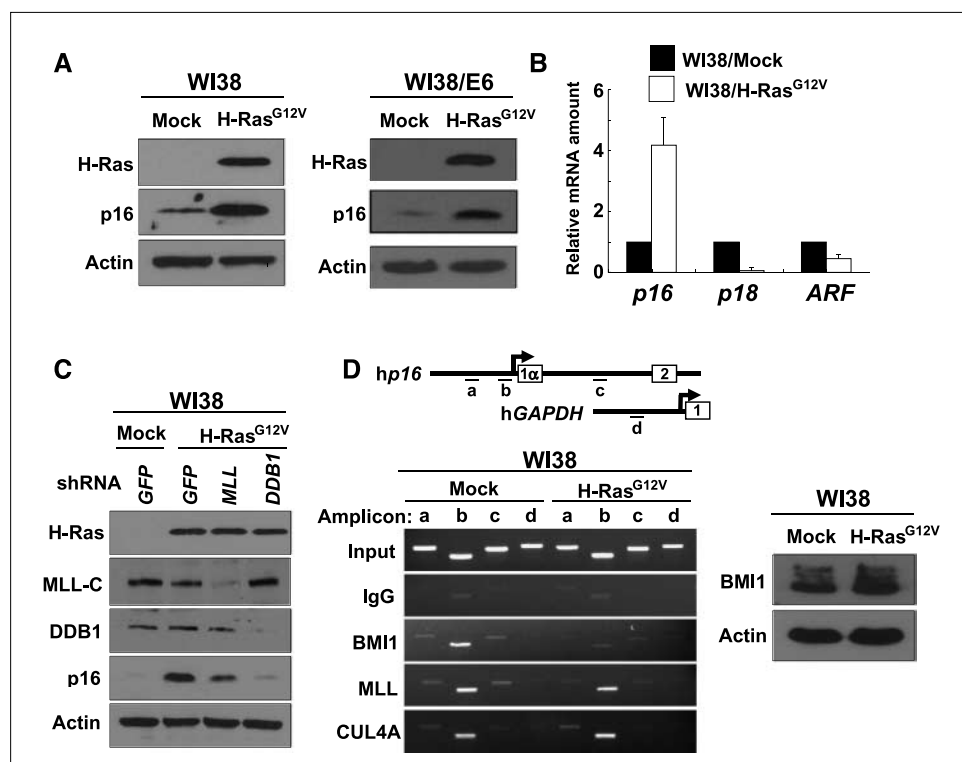
Silencing DDB1 reduces H3K4 trimethylation of *p16* promoter.

To explore the functional relationship between DDB1, MLL1, and polycomb complexes in the regulation *p16* gene expression, we first determined whether silencing DDB1 in WI38/E7 cells would restore the binding of BMI1 *p16* promoter. After knocking down DDB1 (Fig. 4A), there was still no detectable binding of BMI1 to *p16* in WI38/E7 cells (Fig. 4B), indicating that dissociation of BMI1 from *p16* promoter is not due the competitive occupancy by DDB1-CUL4A. This conclusion is consistent with the notion that polycomb-mediated repression of *p16* expression is dominant over DDB1-mediated *p16* activation.

We next determined whether the binding of CUL4A and MLL1 to *p16* promoter depends on each other by ChIP assay after silencing either MLL1 or DDB1 in WI38/E7 cells. These experiments showed that knocking down MLL1 (Fig. 4A), while nearly completely abolished the binding of MLL1 to *p16* promoter, had no appreciable effect on the binding of CUL4A to *p16* (Fig. 4C). Likewise, silencing of DDB1 did not significantly affect the binding of MLL1 to *p16*. These results show that these two complexes bind to *p16* independently as oppose to one recruiting the other. We note that silencing of DDB1 did not significantly affect the binding of CUL4A to *p16* promoter, suggesting that the binding of CUL4A to *p16* is not depending on DDB1. Finally, we determined H3K4 trimethylation in WI38/E7 cells after knocking down DDB1 and found that knocking down DDB1 by shRNA resulted in a decrease of H3K4 methylation of the *p16* locus by >50% (Fig. 4D), suggesting that DDB1-CUL4A facilitates, or may even be required for, MLL1-mediated H3K4 methylation at the *p16* locus.

CUL4-DDB1 and MLL1 are required for oncogene-induced *p16* expression. *p16* gene expression is activated by a variety of oncogenes, causing stable cell cycle arrest to protect cells from

Figure 5. CUL4-DDB1 and MLL1 are required for oncogene-induced *p16* expression. *A* and *B*, WI38 or WI38/E6 cells were infected with empty (Mock) or oncogenic H-Ras^{G12V}-expressing retroviruses and selected by puromycin treatment. The levels of individual protein (*A*) and mRNA (*B*) levels were determined by immunoblotting and Q-RT-PCR, respectively. *C*, WI38 cells were infected with the retroviruses expressing empty (*Mock*) or H-Ras^{G12V} and shRNA targeting *GFP*, *MLL1*, or *DDB1*, selected by puromycin treatment for 3 d and harvested 8 d after initial infection. The levels of individual protein were determined by immunoblotting. *D*, a schematic representation of the human *p16* and *GAPDH* gene loci and amplicons (*a*, *b*, *c*, and *d*) used for ChIP assays. Antibodies against BMI1, MLL1, CUL4A, and IgG control were used in the ChIP assay. PCR was carried out using primers for each amplicon as indicated. The BMI1 protein level was determined by immunoblotting.



hyperproliferative stimulation (15). Transduction of oncogenic H-Ras^{G12V} in WI38 cells resulted in a nearly 6-fold increase of *p16* mRNA, whereas both *p18* and *ARF* levels were decreased (Fig. 5*A* and *B*). The latter is consistent with others' findings that there is a difference between human and murine cells in the regulation of *ARF* in response to Ras-instigated oncogenic insults (28). Oncogenic H-Ras^{G12V} also induced *p16* in WI38/E6 cells (Fig. 4*A*), indicating that induction of *p16* by oncogenic H-Ras^{G12V} is not dependent on the function of p53.

Knocking down MLL1 reduced oncogenic H-Ras^{G12V}-induced *p16* activation, whereas silencing *DDB1* nearly completely abolished *p16* induction by oncogenic H-Ras^{G12V} (Fig. 5*C*). These results identify two novel and critical components, DDB1 and MLL1, on the pathway of oncogene-induced *p16* activation. ChIP assay showed that H-Ras^{G12V} expression completely disrupted the binding of BMI1 to the *p16* locus (Fig. 5*D*), although the steady-state level of the BMI1 protein is slightly increased. The amounts of MLL1 or CUL4A binding to the *p16* locus, on the other hand, were unchanged, again suggesting that PRC-mediated repression of *p16* acts dominantly over the DDB1-CUL4 and MLL1-mediated *p16* activation and that removal of PRC-imposed repression is a prerequisite for *p16* induction.

Discussion

In this study, we identified two novel enzymatic activities, an E3 ubiquitin ligase mediated by the DDB1-CUL4-ROC1 complex and a histone methyltransferase provided by the MLL1 catalytic complex and a structural complex consisting of WDR5-RbBP5-Ash2L that are required for *p16* activation. We further show that a key component from each enzyme complex, DDB1 and MLL1, are required for oncogene-induced activation of *p16*. The results

presented here provide the new insight into the epigenetic control of *p16* gene expression. These findings represent a significant advance from and are mechanistically linked with previously established epigenetic control of *p16* repression by polycomb. Very recently, Kia and colleagues (29) reported that in malignant rhabdoid tumor (MRT)-derived cells that reactivation of *p16* expression by the reintroduction of SWI/SNF chromatin-remodeling complex is associated with the dissociation of PRC from and recruitment of MLL1 to the *p16* promoter. The results reported in this study provide an additional support in the setting of primary nontransformed cells that MLL1 binds to and is directly involved in *p16* activation. It is worthy noting that in these MRT cells, PRC can be displaced by SWI/SNF, exhibiting a "recessive" feature to the SWI/SNF complex. This is clear different from the dominant nature of PRC over both MLL1 and CUL4-DDB1 as we have observed. It will be important to determine whether this dominance of SWI/SNF chromatin remodeling complex over PRC presents a special case in MRT cells (30) and how SWI/SNF activity affect the recruitment of both MLL1 histone methyltransferase and CUL4-DDB1 ubiquitin ligase complexes to the *p16* promoter.

The detailed biochemical mechanisms underlying the function of DDB1-CUL4A and MLL1 in *p16* activation remain to be determined, including in particular the identity of the substrate(s) of DDB1-CUL4-ROC1 ubiquitin ligase and how the ubiquitination(s) affects MLL1-mediated H3K4 trimethylation. Ohtani and colleagues (31) previously reported that ectopic expressed transcription factors Ets1 and Ets2 also bind to and activate *p16* in senescent human fibroblasts and that Ras-induced *p16* is diminished by the ectopic expression of Id1, a dominant-negative regulator of Ets factor. The functional relationship between Ets-Id and DDB1-CUL4/MLL1 in mediating *p16* activation remains to be investigated. p38 mitogen-activated protein kinase (MAPK), a

major cellular stress checkpoint mediator, has also been previously implicated in *p16* induction by Ras oncogene (32, 33). Whether activation of p38 MAPK leads to derepression of *p16*, through either induction of cyclin D and thus inactivation of RB or phosphorylation of BMI1 and thus dissociation of polycomb from chromatin (34), is also an interesting issue that is yet to be investigated.

A significant issue concerns whether the well-established DNA damage checkpoint pathway, which is mediated by ATM/ATR, CHK1/CHK2, and p53 and was recently shown to be required for oncogene-induced senescence (35–38), is linked with the DDB1-CUL4-MLL1 in oncogene-induced *p16* activation. It has long been noticed that inactivation of p53 does not affect *p16* induction (reviewed in ref. 1). Our data showed that inactivation of p53 has no detectable effect on Ras-induced *p16* expression (Fig. 5A). These observations, together with the dissociated kinetics between p53 activation (as early as within 24 hours) and *p16* induction by either DNA damage or by oncogenic Ras (more than a week), argue against the possibility that Ras signals a component of either DDB1-CUL4-ROC1 ligase or MLL1 methyltransferase in a p53-

dependent manner. Our extensive efforts have thus far failed to detect any significant effect of ATM, ATR, CHK1, or CHK2 in Ras-mediated *p16* activation, raising the possibility that DDB1-CUL4, WDR5-RbBP5-Ash2L, and MLL1 complexes constitute a novel pathway that mediates *p16* activation during oncogenic checkpoint response and cell aging and that is repressed during normal growth of young cells by the PRCs.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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