DDB1-CUL4 and MLL1 Mediate Oncogene-Induced p16\(^{INK4a}\) Activation

Yojiro Kotake,\(^1\) Yaxue Zeng,\(^2,1\) and Yue Xiong\(^{1,2}\)

\(^1\)Lineberger Comprehensive Cancer Center and \(^2\)Department of Biochemistry and Biophysics, Program in Molecular Biology and Biotechnology, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina; and \(^3\)Department of Biochemistry, 1,3Hamamatsu University School of Medicine, Higashi-ku, Hamamatsu, Japan

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 (Ab-4; Neomarkers), MLL1 (BETHYL Laboratories, Inc.), RbBP5 (Mammalian 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 (MADEAPRKGSF-SALYRTGNG) 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 were 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. Retrovirus encoding shRNAs silencing INK4a, MLL1, WDR5, Menin, and control GFP were constructed by ligating respective oligonucleotides (MLL1, GGATCGTTAAGTGGTCATAAA; WDR5, CACCTGTAAGCCTAACTA; RbBP5, GAGCCGAGATGGTCATAAA; Menin, GTCGCAGATGCAGATGAAG; GFP, GCTCACCGAGCGGACCA) 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 \(\mu\)g was used for cDNA synthesis primed with Oligo(dt)\(_{18}\) 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 \(\times\) 10\(^6\) 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
[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 (pH 7.9), 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 p18INK4c mRNA, but not two other INK4 genes (p15INK4b, p19INK4d), were also decreased. This result is consistent with previous findings that the p18 locus is bound and transcriptionally activated by a MLLI 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 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 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.

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 for the more physiologic increase in p16 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 MLL1 to p16 promoter. After knocking down DDB1 (Fig. 4A), there was still no detectable binding of MLL1 to p16 in WI38/E7 cells (Fig. 4B), indicating that dissociation of MLL1 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. 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 siRNA 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 senescence. ChIP assay showed that CUL4A bound 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 MLL1 to p16 promoter. After knocking down DDB1 (Fig. 4A), there was still no detectable binding of MLL1 to p16 in WI38/E7 cells (Fig. 4B), indicating that dissociation of MLL1 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. 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 siRNA 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
Oncogene Activates p16 via DDB1-CUL4 and MLL1

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 in the context 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 complex over PRC presents a special state of PRC that is required for PRC-imposed repression is a prerequisite for p16 induction.

Table 1

<table>
<thead>
<tr>
<th>p16</th>
<th>H-Ras</th>
<th>Actin</th>
<th>MLL1</th>
<th>DDB1</th>
<th>shRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. WI38</td>
<td>Mock</td>
<td>H-Ras&lt;sup&gt;G12V&lt;/sup&gt;</td>
<td>p16</td>
<td>Actin</td>
<td></td>
</tr>
<tr>
<td>B. WI38/E6</td>
<td>Mock</td>
<td>H-Ras&lt;sup&gt;G12V&lt;/sup&gt;</td>
<td>p16</td>
<td>Actin</td>
<td></td>
</tr>
<tr>
<td>C. WI38</td>
<td>Mock</td>
<td>H-Ras&lt;sup&gt;G12V&lt;/sup&gt;</td>
<td>MLL1</td>
<td>DDB1</td>
<td></td>
</tr>
<tr>
<td>D. WI38</td>
<td>H-Ras&lt;sup&gt;G12V&lt;/sup&gt;</td>
<td>H-Ras&lt;sup&gt;G12V&lt;/sup&gt;</td>
<td>BMI1</td>
<td>Actin</td>
<td></td>
</tr>
</tbody>
</table>

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<sup>G12V</sup>-expressing retroviruses and selected by puromycin treatment. The levels of individual protein (A) and mRNA (B) were determined by immunoblotting and Q-RT-PCR, respectively. C, WI38 cells were infected with the retroviruses expressing empty (Mock) or H-Ras<sup>G12V</sup> 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 primes for each amplicon as indicated. The BMI1 protein level was determined by immunoblotting.

hyperproliferative stimulation (15). Transduction of oncogenic H-Ras<sup>G12V</sup> in WI38 cells resulted in a nearly 6-fold increase of p16 mRNA, whereas both p18 and ARF levels were decreased (Fig. 5A 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<sup>G12V</sup> also induced p16 in WI38/E6 cells (Fig. 4A), indicating that induction of p16 by oncogenic H-Ras<sup>G12V</sup> is not dependent on the function of p53.

Knocking down MLL1 reduced oncogenic H-Ras<sup>G12V</sup>-induced p16 activation, whereas silencing DDB1 nearly completely abolished p16 induction by oncogenic H-Ras<sup>G12V</sup> (Fig. 5C). 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<sup>G12V</sup> expression completely disrupted the binding of BMI1 to the p16 locus (Fig. 5D), 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.

www.aacrjournals.org 1813 Cancer Res 2009; 69: (5). March 1, 2009
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 BM1 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 complex in oncogene-induced p16 activation. It has long been noticed 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–RBP5–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.

Acknowledgments

Received 7/17/2008; revised 10/28/2008; accepted 11/21/2008; published OnlineFirst 02/10/2009.

Grant support: NIH grant CA63877 (Y. Xiong).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Ned Sharpless and Yi Zhang for helpful discussions and Matt Smith and Sarah Jackson for reading the manuscript.

References