PLU-1 Is an H3K4 Demethylase Involved in Transcriptional Repression and Breast Cancer Cell Proliferation

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SUMMARY

Posttranslational modification of chromatin by histone methylation has wide-ranging effects on nuclear function, including transcriptional regulation, maintenance of genome integrity, and epigenetic inheritance. The enzymes utilized to place histone methylation marks are well characterized, but the identity of a histone demethylation system remained elusive until recently. The discovery of histone demethylase enzymes capable of directly removing methyl groups from modified lysine residues has demonstrated that histone methylation is a dynamic modification. The most extensive family of histone demethylase enzymes identified so far contains a JmjC domain and catalyzes demethylation through a hydroxylation reaction. Here, we identify PLU-1, a transcriptional repressor implicated in breast cancer, as a histone demethylase enzyme that has the ability to reverse the trimethyl H3K4 modification state. Furthermore, we reveal that PLU-1-mediated H3K4 demethylase activity plays an important role in the proliferative capacity of breast cancer cells through repression of tumor suppressor genes, including BRCA1.

INTRODUCTION

Recent studies have demonstrated that covalent histone modifications, particularly methylation, play important roles in transcription regulation, genome integrity, and epigenetic inheritance (Martin and Zhang, 2005). Most of the enzymes responsible for targeting genomic histone lysine methylation contain a SET domain and are highly conserved from yeast to human. Specific histone lysine methylation marks are commonly associated with either the active or the repressed state. For example, histone H3K4, K36, and K79 methylation are generally associated with actively transcribed genes, whereas H3K9, K27, and H4K20 methylation usually mark silenced genes (Martin and Zhang, 2005). Recently, several conserved protein domains have been identified that recognize histone lysine methylation to elicit functional effects. These include the chromodomain, PhD domain, WD40 repeat, and Tudor domain (Zhang, 2006). Methyl-lysine recognition domains permit functional interpretation of histone methylation marks as is exemplified by the transcriptional repressor heterochromatin protein 1 (HP1), which utilizes a chromodomain to bind chromatin containing H3K9 methylation and potentiate the repressed state (Bannister et al., 2001; Lachner et al., 2001).

Until recently, histone methylation was considered a static modification, but the identification of histone demethylase enzymes has revealed that this mark is dynamically regulated (Shi et al., 2004; Tsukada et al., 2006). Histone demethylases have the capacity to regulate not only the modification itself but also function indirectly to antagonize binding of effector proteins to modified chromatin. This is exemplified by JHDM3A/JMJD2A, which can displace HP1 from chromatin by removing the H3K9 methylation mark that HP1 recognizes (Cloos et al., 2006; Fodor et al., 2006; Klose et al., 2006b). An extensive family of proteins from yeast to human contain the JmjC domain, which has recently been characterized as a histone demethylase signature motif (Klose et al., 2006a). Thus far, the JmjC domain-containing family of enzymes has been shown to catalyze removal of H3K9 and H3K36 methylation. There are roughly 30 JmjC domain-containing proteins in mouse and human, suggesting enzymes that target other methylation sites are likely to be found within this protein family.

The biological importance of the histone methylation system in normal development and disease has become more clear through functional analysis of enzymes...
involved in placing these modifications and detailed examination of genomic histone methylation profiles. For example, genetic studies in fly and mouse indicate that histone H3K27 methylation plays important roles in early development through the regulation of HOX gene expression (Cao et al., 2002; Pasini et al., 2004; Ringrose and Paro, 2004). In addition, analysis of histone modifications in cancer has revealed that histone methylation and histone methyltransferase enzymes contribute to the process of malignancy (Fraga et al., 2005; Schneider et al., 2002; Varambally et al., 2002). More recently, the JHDM3C/GASC1 demethylase was shown to contribute to the proliferative capacity of certain cancer cell lines, although the contribution of histone demethylation to this process remains to be examined (Cloos et al., 2006).

To identify additional histone demethylases, we have utilized a bioinformatic approach to narrow down the number of JmjC domain-containing proteins that are potentially enzymatically active based on conservation within the JmjC domain (Klose et al., 2006a). To identify additional histone demethylase enzymes, we took a candidate approach within the seven subfamilies of the JmjC domain-containing proteins. Here, we identify and characterize PLU-1, a JARID1 family member previously linked to breast cancer (Lu et al., 1999), as an H3K4 demethylase. PLU-1 and its associated H3K4 demethylase activity play important roles in the proliferation of breast cancer cells through direct transcriptional repression of tumor suppressor genes that include BRCA1.

RESULTS

JARID1 Proteins Are Potential Histone Demethylases
There are four JARID1 proteins in mouse and human: RBP2/JARID1A, PLU-1/JARID1B, SMCX/JARID1C, and SMCY/JARID1D. All four JARID1 proteins contain at least one JmjN, BRIGHT, PHD, JmjC, and C5HC2-zinc-finger (ZF) domain (Figure 1A). Recently, the JmjC domain has been shown to catalyze Fe(II)/α-ketoglutarate (α-KG)-dependent hydroxylation reactions that lead to histone demethylation (Tsukada et al., 2006). Bioinformatic analysis of the JmjC domain in JARID1 proteins indicates that predicted Fe(II) and α-ketoglutarate (α-KG) binding sites are conserved (Figure 1B), suggesting that these proteins may constitute functional hydroxylase enzymes. In addition, the
documented role of JARID1 proteins in transcription regulation suggests these proteins are likely histone demethylases. RBP2/JARID1A and PLU-1/JARID1B are the most thoroughly characterized JARID1 family members, and both have documented roles in cancer cell proliferation (Benevolenskaya et al., 2005; Lu et al., 1999). Because PLU-1 is abnormally expressed in breast cancer cell lines and tissues (Barrett et al., 2002; Lu et al., 1999; Madsen et al., 2002), we sought to examine whether PLU-1 is a histone demethylase.

**PLU-1/JARID1B Is an H3K4 Demethylase**

To determine if PLU-1 is an active histone demethylase, full-length Flag-tagged human PLU-1 was expressed in SF9 cells and purified by affinity chromatography (Figure S1 in the Supplemental Data available with this article online). To determine whether PLU-1 is an active histone demethylase, recombinant PLU-1 was incubated with histone substrates containing radioactively labeled methyl groups corresponding to several characterized methyl-lysine sites on histones H3 and H4 (Figure 1C). Histone demethylase activity was monitored by release of the demethylated product formaldehyde. Release of labeled formaldehyde was only observed in reactions containing histone substrates labeled with a mutant SET7 (SET7 Y245A) (Figure 1C), which has the capacity to methylate H3K4 to the di- and trimethyl states (Xiao et al., 2003). Consistent with the notion that the enzymatic activity is intrinsic to PLU-1, a dose-dependent formaldehyde release was observed on H3K4 substrates (Figure 1D). Furthermore, immunoprecipitation of transiently transfected mutant PLU-1 with a change in the Fe(II) binding site (H499Y) followed by enzymatic activity assays demonstrated that the H499Y mutation abrogated PLU-1 demethylase activity (Figure 1E).

Currently, lysine-specific histone demethylase 1 (LSD1) is the only characterized enzyme capable of removing H3K4 methylation (Shi et al., 2004). LSD1 differs mechanistically from the JmjC domain-containing histone demethylases, as its activity is limited to the me1 and me2 modification states due to the requirement of a protonated nitrogen on the lysine amine group to initiate demethylation. The fact that LSD1 targets H3K4me1/me2 left open the possibility that H3K4me3 was an irreversible modification state. Given that PLU-1 does not target H3K4me1 containing substrates generated by SET7 (Figure 1C), we sought to determine if PLU-1 had the capacity to remove H3K4me3. To this end, we incubated recombinant PLU-1 with histone peptides corresponding to the N-terminal tail of histone H3 containing me3, me2, or me1 modifications at the lysine four position. Mass spectrometric analysis demonstrates that PLU-1 is able to demethylate both H3K4me3 and H3K4me2, but not H3K4me1 (Figures 1F, 1G, and data not shown). Interestingly, the demethylation reaction does not appear to be processive, as no H3K4me1 or H3K4me0 was generated when an H3K4me3 peptide was used as a substrate. The above results collectively allow us to conclude that PLU-1 is an H3K4-specific histone demethylase that can target removal of the H3K4me3 modification state.

**PLU-1 Targets H3K4 Demethylation In Vivo**

To verify that PLU-1 also targets H3K4 demethylation in vivo, PLU-1 was overexpressed in U2OS cells and histone methylation was analyzed by using modification-specific antibodies. Consistent with mass spectrometry analysis demonstrating that PLU-1 is an H3K4me3 demethylase (Figure 1F), overexpression of PLU-1 resulted in a clear reduction of H3K4me3 staining (Figure 2A, top panels). This effect is dependent on the PLU-1 demethylase activity, as overexpression of the enzymatic-defective H499Y mutant has no noticeable effect on the H3K4me3 levels (Figure 2A, bottom panels). In addition to H3K4me3, overexpression of PLU-1 also resulted in a significant decrease on the H3K4me2 and me1 levels (Figures 2B and 2C). However, overexpression of PLU-1 had no effect on the other histone methylation sites, including H3K27me3 (Figure 2D) and H3K9me3 (Figure 2E). It is interesting to note that PLU-1 efficiently catalyzed removal of the H3K4me1 in vivo (Figure 2C), although recombinant PLU-1 can only demethylate H3K4me3 and H3K4me2 when analyzed in vitro (Figure 1). This observation suggests that the ability of PLU-1 to demethylate H3K4me1 may rely on additional factors in vivo.

In addition to the JmjC domain, PLU-1 also contains JmjN, BRIGHT, PHD, and C5HC2-ZF domains (Figure 1A). Previous studies have revealed that domains in addition to JmjC can significantly contribute to enzymatic activity (Fodor et al., 2006; Klose et al., 2006b; Tsukada et al., 2006; Yamane et al., 2006). To test whether other domains within PLU-1 also contribute to demethylase activity, we generated a series of deletion mutants (Figure 3A) and monitored enzymatic activity by analyzing H3K4me3 levels in transfected cells. Removal of the C-terminal PHD domains did not affect PLU-1 demethylase activity (Figure 3B). Further deletion of the C-terminal C5HC2-ZF domain resulted in both nuclear and cytoplasmic localization and caused a complete loss of demethylase activity (Figure 3C). In JHDM3A/JMJD2A, the JmjN domain is required for efficient enzyme function (Fodor et al., 2006; Klose et al., 2006a). To examine whether PLU-1 also requires the JmjN domain for enzymatic activity, the JmjN domain was deleted within the context of the full-length protein (Figure 3A). Although deletion of the JmjN domain did not affect nuclear localization, it completely abrogated H3K4 demethylase activity (Figure 3D). In PLU-1, the JmjN and JmjC are intervened by BRIGHT and PHD domains (Figure 3A). Deletion of these domains had no effect on PLU-1 nuclear localization but did result in loss of demethylase activity (Figure 3E and F). Given that neither the BRIGHT nor the PHD domains are absolutely required for demethylase activity in other JmjC domain-containing proteins, deletion of these intervening domains may alter the spatial arrangement between the JmjN and JmjC domains and thus inhibit accurate folding of the functional enzyme.
PLU-1 Contributes to MCF-7 Cell Proliferation by Facilitating G1 Progression

PLU-1 was initially isolated as a transcript overexpressed in human breast cancer cell lines, and analysis of its expression in breast cancer cell lines and primary breast cancers suggests that 90% of invasive ductal carcinomas express PLU-1 (Barrett et al., 2002; Lu et al., 1999). To examine the role of PLU-1 in the proliferative capacity of MCF-7 cells, a stable shRNA knockdown strategy was utilized to reduce PLU-1 expression. Two knockdown clones (KD1 and KD2) that target two different regions of PLU-1 mRNA were generated. Both knockdown lines showed significant reduction in the levels of PLU-1 expression as accessed by western blot analysis (Figure 4A) and real-time RT-PCR (Figure 4H). Although PLU-1 has several related homologs (Figure 1A), knockdown of PLU-1 does not affect the expression of a closely related family member, JARID1A/RBP2 (Figure S2), indicating the knockdown is specific.

To examine whether reduced PLU-1 levels affect MCF-7 cell proliferation, the growth rate of the knockdown cell lines was compared to the control cell lines derived from transfection of empty vectors (Figure 4B). Both PLU-1 knockdown cell lines exhibited a slower growth rate when compared to that of the control cell lines, indicating that PLU-1 expression is directly related to the proliferative capacity of MCF-7 cells. Consistent with this observation, anchorage-independent soft-agar colony-formation analysis demonstrates that PLU-1 knockdown inhibits colony formation (Figure 4C). Because reduction of PLU-1 levels in MCF-7 cells decreased their colony-forming capacity, PLU-1 might contribute to the transformed phenotype of MCF-7. To understand how PLU-1 knockdown leads to the cell-proliferation defects, we performed apoptosis and cell-cycle analysis. No significant difference in the ratio of apoptotic cells is observed when the knockdown cells are compared with control cells (Figure 4D). FACS analysis demonstrates that knockdown of PLU-1 reduces the S phase cell population, from 41% to 34%, with a concomitant increase in the G1 cell population from 45% to 53%, whereas the G2/M cell population remained essentially unchanged (Figure 4E). These data suggest a G1 promoting activity of PLU-1. Consistent with this finding, 45% of control cells have entered the S phase at 24 hr, but only 34% of PLU-1 knockdown cells have reached S phase at the same time after addition of serum to serum-starved MCF-7 cells (Figure 4F). Based on these results, we conclude that PLU-1 positively regulates the cell cycle by facilitating the G1 to S transition.

PLU-1 Represses Negative Regulators of Cell Growth

PLU-1 functions as transcriptional repressor when tethered to an artificial reporter gene in transient transfection.
assays, suggesting that PLU-1 may function to repress genes related to proliferation of MCF-7 cells (Tan et al., 2003). To examine this possibility, the gene expression levels of a series of tumor suppressor genes previously linked to breast cancer were examined in PLU-1 knockdown cell lines. Because the sole JARID1 homolog in Drosophila, Lid, is a trithorax group protein (Gildea et al., 2000), we also analyzed the effect of PLU-1 knockdown on the expression of some HOXA genes. Of the genes analyzed, an increase in expression of several genes, including 14-3-3s, BRCA1, CAV1, and HOXA5, was observed in knockdown cell lines as assessed by conventional and real-time RT-PCR (Figures 4G and 4H). Upregulation of these genes was observed in both knockdown cell lines, verifying that expression differences are caused by PLU-1 knockdown. None of the candidate genes examined in PLU-1 knockdown cells showed reduced gene expression, in support of the observation that PLU-1 is a transcriptional repressor (Tan et al., 2003).

**Knockdown of PLU-1 Results in Increased H3K4me3 Levels at Target Genes**

To determine if genes affected in knockdown lines are direct targets of PLU-1, chromatin immunoprecipitation (ChIP) was utilized to access association of PLU-1 with the 14-3-3s, CAV1, HOXA5, and BRCA1 genes (Figures 5A, 5C, and 5E). Toward this end, four regions in each gene that are highly conserved between human and mouse were chosen for PLU-1 ChIP analysis. This analysis demonstrated that PLU-1 was efficiently immunoprecipitated at three of the four genes analyzed (Figures 5A, 5C, and 5E), demonstrating that PLU-1 is directly associated with these target genes. However, no binding of PLU-1 to the 14-3-3s gene was detected (data not shown), indicating that PLU-1 may regulate 14-3-3s expression indirectly. It is interesting to note, of the three target genes identified, PLU-1 bound to each of them in a region close to the transcriptional start site, suggesting its repressive effects may be related to transcriptional initiation.

To determine the consequence of PLU-1 binding, H3K4 methylation levels were analyzed at PLU-1-associated regions in control and PLU-1 KD cells (Figures 5B, 5D, and 5F). In PLU-1 KD cells, all three target genes showed reduced levels of PLU-1 occupancy, which correlated with increases in H3K4me3 levels, but the H3K4me2 and me1 levels were unaffected. The effects on H3K4me3 methylation were specific to PLU-1-associated regions, as portions of the gene not associated with PLU-1 showed no change in H3K4me3 levels in response to PLU-1 KD. Surprisingly, the levels of H3K4me2 and me1 were not

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**Figure 3. Multiple Domains in PLU-1 Contribute to Its Histone Demethylase Activity**

(A) Schematic representation of wild-type and mutant PLU-1 proteins with their localization pattern and demethylase activity against H3K4me3 indicated right. For the demethylase activity, a plus sign (+) represents active and a minus sign (−) represents inactive.

(B–F) Various mutant PLU-1-MYC proteins were expressed in U2OS cells, and histone modification levels were analyzed by immunofluorescent staining with H3K4me3-specific antibodies. Arrows indicate PLU-1-transfected cells. Only the C-terminal portion of PLU-1 is dispensable for its H3K4me3 demethylase activity (B). Shown are representative fields for each transfection.
Figure 4. PLU-1 Knockdown Impairs Cell Proliferation through G1 Arrest and Upregulation of Certain Tumor Suppressor Genes

(A) Two PLU-1 shRNA knockdown cell lines (KD1 and KD2) were generated by stable transfection of MCF-7 cells. Both cell lines show greater than 90% knockdown at the protein level as assessed by western blot analysis for PLU-1 (top panel). $\alpha$-tubulin serves as a loading control (bottom panel).

(B) The growth rate of PLU-1 knockdown cell lines was compared to control cell lines over a 5 day period. PLU-1 knockdown lines showed a marked decrease in proliferation compared to the control cells. Shown is the average of three independent experiments with error bars. An asterisk (*) indicates both KD lines are statistically significant ($p < 0.05$) when compared with Ctrl1.

(C) Wild-type and PLU-1 knockdown MCF-7 cells were assayed for their ability to proliferate and form colonies in soft agar. Shown are representative fields from control (Ctrl) and knockdown (KD1 and KD2) cells after 21 days of culture. Scale bar, 0.2 mm.

(D) PLU-1 knockdown does not induce apoptosis in MCF-7 cells. Apoptotic cells were detected by flow cytometry using FITC-conjugated Annexin V staining. Shown is the average of three independent experiments with error bars.

(E and F) Flow-cytometry analysis of cell-cycle distribution. Asynchronized (E) or serum-starvation synchronized (F) MCF-7 cells were stained with PI and analyzed by flow cytometry. Shown are representative results from three independent experiments.

(G) The transcript levels of several breast cancer-associated genes and Hox genes were analyzed in control and KD cell lines by using RT-PCR. Several genes implicated in breast cancer cell proliferation were specifically upregulated in knockdown cells, including 14-3-3$\alpha$, BRCA1, CAV1, and HOXA5.

(H) Genes showing reactivation by conventional RT-PCR were analyzed by real-time RT-PCR to quantify fold increase in gene expression compared to control lines.

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significantly affected in the KD cell lines, suggesting that although PLU-1 can target all three H3K4 methylation states (Figure 2) it preferentially regulates the level of H3K4me3 at target genes in MCF-7 cells. In contrast to the gene-specific effect described above, knockdown of PLU-1 does not affect global H3K4 methylation levels (Figure S3), likely due to the existence of multiple H3K4 demethylases in MCF-7 cells.

PLU-1-Mediated H3K4 Demethylation Is Required for Repression of Target Genes

To examine whether PLU-1 relies on its H3K4 demethylase activity for transcriptional repression, wild-type and catalytically deficient PLU-1 were expressed in 293T cells and the expression of PLU-1 target genes was analyzed by real-time RT-PCR (Figure 6A). Expression of wild-type protein had little effect on gene expression with target genes remaining repressed. Expression of a catalytically deficient mutant of PLU-1 resulted in increased target-gene expression (Figure 6A), demonstrating that histone demethylation is directly linked to PLU-1-mediated transcriptional repression. To analyze whether the exogenous PLU-1 binds directly to its endogenous target genes, we chose to analyze PLU-1-Myc occupancy on HOXA5 gene by ChIP, which revealed a similar binding pattern to that of endogenous PLU-1 (Figures 6B and 5C). This suggests that the catalytically deficient PLU-1 mutant may alleviate repression by acting in a dominant-negative fashion to inhibit active H3K4 demethylation. To directly examine whether the catalytically deficient PLU-1 mutant functions to alleviate H3K4me3 demethylation, the levels of H3K4 methylation at the PLU-1 binding site of the HOXA5 gene were analyzed in cells expressing wild-type or mutant PLU-1 (Figure 6C). Expression of the wild-type protein resulted in a reduction of H3K4me3, indicating that an increased level of PLU-1 potentiated H3K4me3 demethylation. In cells expressing catalytically deficient PLU-1, there was an increase in H3K4me3 methylation, suggesting a key function of PLU-1 in transcriptional regulation of target genes is to antagonize H3K4 methylation (Figure 6C). Together, these data provide strong evidence that PLU-1 functions to maintain breast cancer cell...
proliferation through a transcriptional repression program relying on its histone demethylase activity.

**PLU-1 Promotes Cancer Cell Proliferation in a Syngeneic Mouse Mammary Tumor Model**

We have demonstrated in the human breast cancer cell line MCF-7 that PLU-1 positively regulates cell proliferation by facilitating G1/S transition (Figure 4). To assess the role of PLU-1 in breast cancer in vivo, we utilized a syngeneic mouse mammary tumor model (Heppner et al., 2000). It has been reported that the 4T1 cells, originally derived from a spontaneous mouse mammary carcinoma in the BALB/c strain (Aslakson and Miller, 1992), can mimic several aspects of the human mammary carcinoma (Pulaski et al., 2000). Recently, the role of STAT3 in breast cancer growth and metastasis has been studied by injection of the 4T1 cells, with or without knocking down of STAT3, into BALB/c mice (Ling and Arlinghaus, 2005). To explore the role of mouse Plu-1 in mammary tumorigenesis in this model, we generated two stable knockdown 4T1 clones (KD1 and KD2) that target different regions of Plu-1 mRNA. Western blot analysis indicates that both knockdown lines showed at least 50% reduction in the levels of Plu-1 expression (Figure 7A). Similar to the observations in the MCF-7 cell line (Figure 4B), knockdown of Plu-1 reduced tumor cell growth. Thus, we conclude that Plu-1 contributes to breast cancer cell proliferation in vivo.

**DISCUSSION**

**H3K4me3 Is Reversible**

Using a candidate approach, we have identified PLU-1 as an H3K4 demethylase that functions both in vitro and in vivo to remove the H3K4me3 modification state. H3K4me3 correlates strongly with active gene expression. In budding yeast, this modification is specifically enriched in the 5' end of actively transcribed genes and is involved in transcriptional initiation and elongation (Zhang et al., 2005). Upon transcriptional activation, H3K4me3 levels rapidly increase but are swiftly lost following attenuation of transcription (Zhang et al., 2005). This loss of H3K4me3 could be explained by histone eviction or replacement, but the identification of an H3K4me3 demethylase, which has a single ortholog in budding yeast, provides a direct enzymatic mechanism by which H3K4 methylation can be rapidly removed and potentially contribute to transcriptional silencing. In mammals, the dynamics of H3K4 methylation are less well characterized, but our observations suggest that PLU-1 can maintain the repressed state of certain genes through H3K4 demethylation. One aspect of transcriptional regulation where H3K4 demethylation could potentially have important roles is in the regulation of inducible genes whose protein product is tightly controlled and only required in the presence of certain cellular stimuli. For example, during hormone-responsive gene activation, there is a rapid increase in H3K4 methylation...
concomitant with gene activation (Dreijerink et al., 2006). In the absence of an activating signal, transcriptional repressors are recruited to these inducible genes to reset the silenced state. It will be interesting to examine whether PLU-1, or related H3K4 demethylases, are involved in re-establishing the silenced state and reversing the H3K4 methylation marks during this phase of active repression.

### Regulation of Breast Cancer-Related Genes by PLU-1-Mediated H3K4 Demethylation

PLU-1 was first identified as a gene upregulated in breast cancer cell lines (Lu et al., 1999). Initial studies characterizing PLU-1 as a transcriptional repressor suggested its abnormal expression in breast cancer lines may contribute to altered transcriptional profiles of breast cancer-related genes. To explore this possibility, we examined the gene-expression profiles of several cancer-associated genes in two independent PLU-1 knockdown cell lines and found that BRCA-1, CAV1, 14-3-3-sigma, and HOXA5 were specifically upregulated in the PLU-1 knockdown cells. In addition to gene expression defects, PLU-1 knockdown cells also displayed a reduced growth rate likely due to a defect in the G1/S transition. It is likely that reduced proliferation in PLU-1 knockdown cells is due to re-expression of genes normally repressed by PLU-1, as all identified target genes have been implicated in inhibition of cell growth. For example, exogenous BRCA1 expression in MCF-7 cells caused substantial reduction in cell growth and failed to support tumor formation when injected into nude mice (Jensen et al., 1996). Like BRCA1, exogenous expression of CAV1 in MCF-7 cells also leads to reduced cell proliferation (Fiucci et al., 2002). Interestingly, exogenous expression of HOXA5 was previously shown to induce p53 expression, leading to apoptosis in breast cancer cells (Raman et al., 2000). In PLU-1 knockdown cells, HOXA5 is upregulated 4-fold but p53 levels are only slightly increased in one of the two knockdown lines. This suggests that the level of HOXA5 upregulation in PLU-1 knockdown cells is not sufficient to result in robust p53 reactivation but does not rule out the possibility that other target genes more sensitive to HOXA5 levels are affected. Together, these observations indicate that PLU-1 positively regulates cell proliferation by repressing the expression of cell-growth inhibitors.

Recent studies indicate that altered expression profiles of histone methyltransferase enzymes or abnormal targeting of these enzymes contributes to malignancy (Hammamori et al., 2004; Jones and Baylin, 2007; Okada et al., 2005, 2006). This is exemplified by the oncogenic MLL-AF10 fusion protein, which aberrantly targets DOT1L-mediated H3K79 methylation to the HOXA9 gene, leading to increased gene expression and leukemogenesis (Okada et al., 2005). Based on the prevalence of altered histone methylation profiles in cancer, it seems likely that histone demethylases will also be exploited to circumvent normal cellular homeostasis. In agreement with this hypothesis, MCF-7 breast cancer lines utilize the H3K4 demethylase activity of PLU-1 to repress genes normally involved in control of cell growth. All PLU-1 target genes analyzed show a direct correlation between expression level and the capacity of PLU-1 to regulate promoter-associated H3K4me3 levels. It will be interesting to determine, using global gene expression profiling techniques, whether PLU-1 has a more extensive role in regulating transcription.
in breast cancers. In addition to the MCF-7 human breast cancer cells, we have also demonstrated a role for Plu-1 in murine mammary tumor by using the mouse 4T1/BALB/c immunocompetent tumor model system. If PLU-1 functions as a pivotal regulator of breast cancer proliferation, this could be a potentially important therapeutic target. Because PLU-1 appears to mediate its repressive effect through H3K4 demethylation, compounds targeting its demethylase function may act to inhibit breast cancer cell growth.

Regulation of H3K4 Methylation by JARID1 Family Members

PLU-1 is a member of the JARID1 family of proteins that consists of four members in mouse and human. The other three members of the JARID1 family (JARID1A/RBP2, JARID1C/SMCX, and JARID1D/SMCY) are highly similar to PLU-1, and all have conserved residues within the predicted cofactor binding sites, suggesting that they will function as H3K4 demethylases (Klose et al., 2006a). The sequence similarity and domain architecture conservation between JARID1 family members suggests that some functional redundancy between these proteins is likely to exist, but experimental evidence indicates that individual members also have unique functional properties and divergent expression profiles.

JARID1A/RBP2 was the first characterized member of the JARID1 family and was originally identified by its physical interaction with the tumor suppressor protein pRB (Deleo-Jones et al., 1991). The effect of RBP2 on transcription is context dependent and can function as either a transcriptional repressor or an activator, depending on the specific target gene analyzed (Benevolenskaya et al., 2005). RBP2 is ubiquitously expressed (Fattaey et al., 1993), and RBP2-null mice display impaired cell growth.

Histone Demethylase Assay

The histone demethylase assay was performed essentially as previously described (Tsukada et al., 2006). Briefly, various 3H-labeled histone substrates or histone peptides were incubated with recombinant PLU-1 in histone demethylation buffer (50 mM HEPES-KOH [pH 7.5], 70 mM Fe[NO3]3[S04]2, 1 mM α-ketoglutarate, and 2 mM ascorbate) at 37°C for 1–3 hr. Demethylation was analyzed by the NASH method and mass spectrometry. For detection of 3H-labeled formaldehyde, a modified NASH method (Kleeberg and Klinger, 1982) was used. After TCA precipitation, an equal volume of NASH reagent (3.89 M ammonium acetate, 0.1 M acetic acid, and 0.2% 2,4-pentanedione) was added into the supernatant and the mixtures were incubated at 37°C for 50 min before extraction with equal volume of 1-pentanol. The extracted radioactivity was measured by scintillation counting. For detection of demethylation with peptide substrates, peptides in the reaction mixtures were desalted on a RP microtip and analyzed by MALDI-TOF as described previously (Tsukada et al., 2006).

Immunostaining

U2OS cells were plated onto glass coverslips in a 12-well plate and cultured for 1 day. Cells were transiently transfected with plasmids by FuGene6. Two days after transfection, cells were washed with PBS and fixed in 4% paraformaldehyde for 10 min. The cells were then washed three times with cold PBS and permeabilized for 5 min with cold PBS containing 0.2% Triton X-100. Permeabilized cells were then washed three times with blocking buffer (1% bovine serum albumin in PBS) and blocked for 30 min before incubation with primary antibodies for 1 hr in a humidified chamber. After three consecutive 5 min washes with PBS, cells were incubated with secondary antibodies for 1 hr before being washed with PBS and stained with 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) in PBS. Cells were washed twice with PBS and then mounted in fluorescent mounting medium (Dako) before being viewed under a Zeiss immunofluorescence microscope.

Generation of Stable PLU-1/Plu-1 Knockdown Cell Lines and Cell-Growth Analyses

MCF-7 and 4T1 cells were cultured in DMEM media supplied with 10% FBS. To generate stable PLU-1/Plu-1 knockdown cell lines, siRNA
vectors were transfected into MCF-7 or 4T1 cells. Stable transfectants were selected in the presence of 1 μg/ml (MCF-7) or 3 μg/ml (4T1) puromycin (Gibco). Cells derived from these transfectants were used for western blotting, real-time PCR, and ChIP analysis. For cell-growth analyses, 3 x 10^4 cells were seeded in 12-well plates. After 24, 48, 72, 96, and 120 hr in culture, cells were trypsinized, collected in triplicate, and counted by trypan-blue staining.

**Cell-Cycle and Apoptosis Analysis**

MCF-7 cells were synchronized by serum starvation for 24 hr and were released by culturing in DMEM media with 10% FBS for 24 hr. After exposure to 10 μM BrdU for 30 min, cells were harvested and fixed in 70% ethanol. Cells were then washed with PBS, resuspended, and incubated with 4N HCL and 0.5% Triton X-100 for 30 min at room temperature. After washing with PBS, cells were neutralized with 0.1 M sodium borate before being labeled with FITC-conjugated BrdU antibody (BD Biosciences) and incubated with 50 μg/ml propidium iodide (Sigma) containing 125 μg/ml RNase (Calbiochem). Cells were then filtered through a nylon mesh (95 μm pore size), and a total of 20,000 stained nuclei were analyzed in a Cyan Flow Cytometer (BD Biosciences). For apoptosis analysis, MCF-7 cells were serum starved for 24 hr and released to DMEM media containing 10% FBS for 24 hr. Cells were harvested and washed with PBS, and then subjected to sequential staining with Annexin V-FITC and propidium iodide according to the manufacturer’s protocol (BD Biosciences) before being analyzed by Cyan Flow Cytometer.

**Soft-Agar Colony-Formation Assays**

MCF-7 cells suspended in 0.35% agar (15,000 cells/dish) were layered to the manufacturer’s protocol (BD Biosciences) before being analyzed by Cyan Flow Cytometer.

**Animals, 4T1 Cell Culture, and Implantation**

All BALB/c mice were purchased from The Jackson Laboratories (Bar Harbor, ME, USA). All procedures involving experimental animals were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee (IACUC). The 4T1 cell line was purchased from the American Type Culture Collection (Manassas, VA, USA) and maintained in DMEM media with 10% FBS. For tumor cell implantation, 1 x 10^7 4T1 cells were injected into mammary fat pad of BALB/c virgin female mice (six mice were injected for each treatment). The tumor size was measured, and the volume was calculated as (length [mm] x width [mm]^2)/2.

**Statistical Analysis**

The data are expressed as means ± SEM. Statistical data of cell growth or tumor volume are analyzed by using a two-tailed Student’s t test. Values of p < 0.05 were considered to be statistically significant.

**Supplemental Data**

Supplemental Data include Supplemental Experimental Procedures, Supplemental References, three figures, and two tables and can be obtained through a nylon mesh (95 μm pore size), and a total of 20,000 stained nuclei were analyzed in a Cyan Flow Cytometer (BD Biosciences). For apoptosis analysis, MCF-7 cells were serum starved for 24 hr and released to DMEM media containing 10% FBS for 24 hr. Cells were harvested and washed with PBS, and then subjected to sequential staining with Annexin V-FITC and propidium iodide according to the manufacturer’s protocol (BD Biosciences) before being analyzed by Cyan Flow Cytometer.

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**References**


