Demethylation of RB Regulator MYPT1 by Histone Demethylase LSD1 Promotes Cell Cycle Progression in Cancer Cells

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Abstract

Histone demethylase LSD1 (also known as KDM1 and AOF2) is active in various cancer cells, but its biological significance in human carcinogenesis is unexplored. In this study, we explored hypothesized interactions between LSD1 and MYPT1, a known regulator of RB1 phosphorylation. We found that MYPT1 was methylated *in vitro* and *in vivo* by histone lysine methyltransferase SETD7 and demethylated by LSD1, identifying Lys 442 of MYPT1 as a target for methylation/demethylation by these enzymes. LSD1 silencing increased MYPT1 protein levels, decreasing the steady state level of phosphorylated RB1 (Ser 807/811) and reducing E2F activity. MYPT1 methylation status influenced the affinity of MYPT1 for the ubiquitin-proteasome pathway of protein turnover. MYPT1 was unstable in murine cells deficient in SETD7, supporting the concept that MYPT1 protein stability is physiologically regulated by methylation status. LSD1 overexpression could activate RB1 phosphorylation by inducing a destabilization of MYPT1 protein. Taken together, our results comprise a novel cell cycle regulatory mechanism mediated by methylation/demethylation dynamics, and they reveal the significance of LSD1 overexpression in human carcinogenesis. *Cancer Res; 71(3); 655–60. ©2010 AACR.*

Introduction

Histone lysine methylation and demethylation play critical roles in regulating chromatin structure. Alteration of chromatin structure is crucial for biological processes such as DNA replication, DNA repair, chromosome recombination, and transcriptional regulation. Although histone lysine methylation was considered to be a static modification until recently, discovery of lysine-specific demethylase 1 (LSD1), the first identified lysine-specific histone demethylase belonging to the flavin-dependent amine oxidase family, revealed that the histone methylation is reversible (1). We recently found that expression levels of LSD1 were significantly higher in cancer

cells than in non-neoplastic cells in various types of tumor (2). Although the role of LSD1 in transcriptional regulation has been well documented (1, 3, 4–9), the biological importance of LSD1 dysregulation in human cancer has not been elucidated. In this study, we identified myosin phosphatase target subunit 1 (MYPT1) as a novel substrate of LSD1, and the demethylation of MYPT1 by LSD1 promoted cell cycle progression through the enhancement of RB1 phosphorylation. This finding implies that methylation/demethylation dynamics of lysine residues can play an important role in functions of a variety of proteins besides histones.

Materials and Methods

In vitro methylation and demethylation assays

To perform *in vitro* methylation assay, SETD7 (Upstate) was incubated with recombinant His-N-MYPT1 (residues 1–500) as a substrate and 2 μCi S-adenosyl-L-[methyl-³H] methionine (GE Healthcare) as a methyl donor in a mixture of 10 μL of methylase activity buffer (50 mmol/L Tris-HCl at pH 8.5), for 1 hour at 30°C. Samples were resolved on a 5% to 20% SDS-PAGE gel (Ready Gel; Bio-Rad), and visualized by fluorography and ponceau S (MP Biomedical) staining. To test the ability of LSD1 to demethylate MYPT1, we firstly methylated MYPT1 with SETD7, and then purified methylated MYPT1 with TALON beads. After dialysis, methylated SETD7 was incubated with recombinant LSD1 or bovine serum albumin (BSA) in demethylation buffer [50 mmol/L Tris-HCl (pH 8.5), 50 mmol/L KCl, 5 mmol/L MgCl₂, and 5% glycerol] for 4 hours

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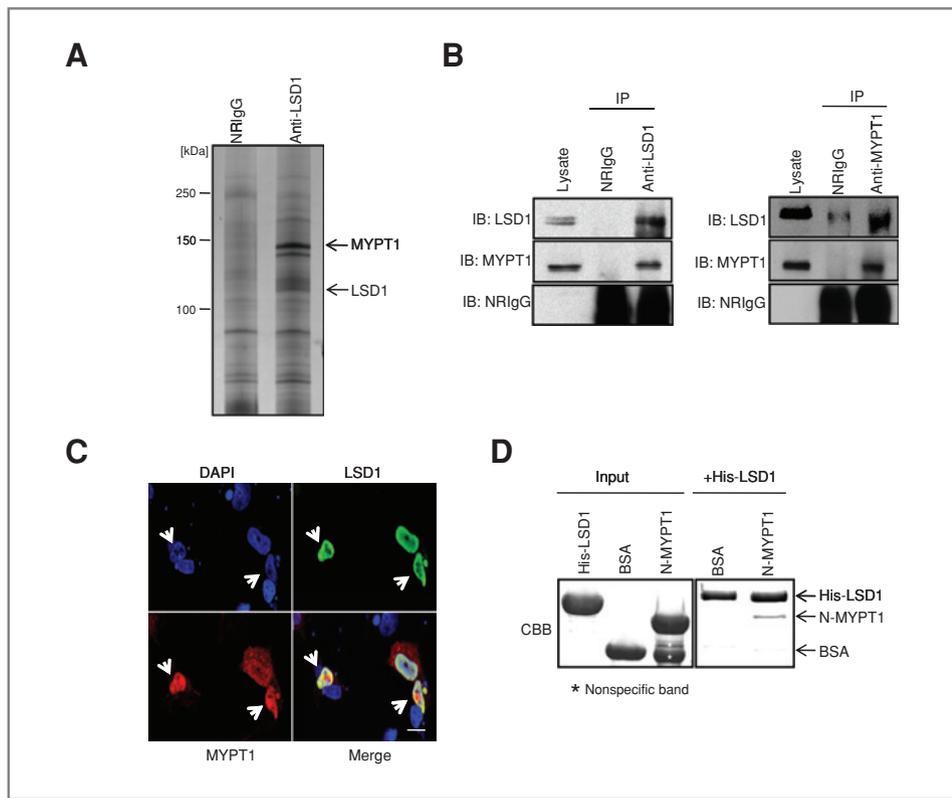
Figure 1. LSD1 interacts with MYPT1. A, interacting protein partners in SBC-5 cell lysates were enriched by anti-LSD1 immunoprecipitation, separated by SDS-PAGE and silver stained. Normal rabbit IgG (NRIgG) was used for negative control. B, immunoprecipitants from lysates of SBC-5 cells using anti-LSD1 and -MYPT1 antibodies were immunoblotted with anti-LSD1 and -MYPT1 antibodies, respectively. NRIgG was used for a negative control. C, immunocytochemical analysis of 293T cells after transfection with FLAG-tagged LSD1 and HA-tagged MYPT1 expression vectors. Scale bar, 20 μm. DAPI, 4′-6-Diamidino-2-phenylindole. D, LSD1 directly interacts with N-terminal region of MYPT1 (1–500), but not BSA. Samples were purified with TALON beads and separated by SDS-PAGE. Proteins were detected by CBB staining.

at 37°C. For MS/MS analysis of LSD1 demethylation, we firstly methylated His-N-MYPT1 with SETD7 for 2 hours at 30°C, sequential adding recombinant LSD1 and incubating for 12 hours at 30°C.

Results and Discussion

To investigate biological significance of LSD1, we have screened a protein(s) interacting with LSD1 through mass spectrometric analysis coupled with immunoprecipitation using anti-LSD1 antibody, and identified MYPT1 as its binding partner (Fig. 1A). MYPT1 relates to myosin phosphatase (MP) activity (10, 11). To verify binding between endogenous LSD1 and MYPT1 proteins, we conducted the immunoprecipitation with anti-LSD1 and -MYPT1 antibodies, and confirmed their endogenous interaction (Fig. 1B). We also found their colocalization in the nucleus (Fig. 1C). To identify the region of LSD1 that interacts with MYPT1, we constructed plasmid clones that were designed to express parts of LSD1 protein and conducted coimmunoprecipitation assay, and found that the central region of LSD1 was required for binding to MYPT1 (Supplementary Fig. S1). Moreover, an *in vitro* binding assay indicated that MYPT1 is directly associated with LSD1 through its N-terminal region (Fig. 1D). Together, these data imply that N-terminal region of MYPT1 directly binds to the central portion of LSD1.

LSD1 was reported to remove the methyl group of histone H3 lysine 4 (H3K4; ref. 1) for which SETD7 was shown to methylate. Because LSD1 was also implied to demethylate a

lysine residue on DNMT1 methylated by SETD7 (12), we suspected a possibility that LSD1 demethylates one or more lysine residues on MYPT1 methylated by SETD7. *In vitro* methylation assay revealed that SETD7 is able to methylate full-length MYPT1 (Supplementary Fig. S2A), and the N-terminal portion of MYPT1 (residues 1–500; Supplementary Fig. S2B), but not the C-terminal region of MYPT1 (data not shown). We subsequently performed an LSD1-dependent demethylase assay using an N-terminal portion of MYPT1 protein after it was methylated by SETD7. The amount of methylated MYPT1 was decreased by addition of LSD1 in a dose-dependent manner (Fig. 2A; Supplementary Fig. S3), indicating demethylase activity on MYPT1. To further confirm the methylation and demethylation of MYPT1 *in vivo*, we conducted an *in vivo* labeling experiment (Fig. 2B) and confirmed that the level of MYPT1 methylation was decreased after treatment with LSD1. Our results strongly imply that MYPT1 can be methylated by SETD7 and demethylated by LSD1 both *in vitro* and *in vivo*.

To identify a site(s) of methylation/demethylation, we performed mass spectrometric analysis after *in vitro* methylation and demethylation assays. Detailed MS/MS analysis indicated that Lys 442 of MYPT1 is monomethylated after treatment with SETD7 (Supplementary Fig. S4). We also analyzed MS data to quantify the amount of methylated peptides (meKTGSYGALAEITASK) and unmethylated peptides (KTGSYGALAEITASK) using MYPT1 samples incubated with SETD7, and subsequent LSD1-dependent demethylation assay (Supplementary Fig. S5). The mass chromatogram data
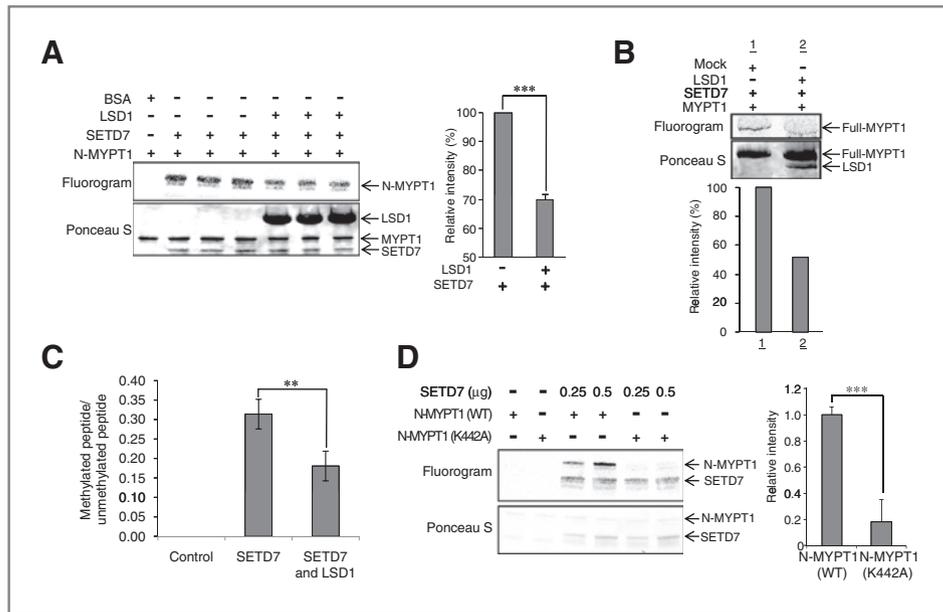
Figure 2. MYPT1 is methylated by SETD7 and demethylated by LSD1. A, *in vitro* demethylation of N-MYPT1 by LSD1. The signal intensity corresponding MYPT1 methylation was quantified by image J. Mean ± SD of 3 independent experiments. *P* values were calculated using Student's *t* test (***, *P* < 0.001). B, *in vivo* methylation/demethylation of MYPT1. 293T cells were transfected with FLAG-tagged MYPT1 and FLAG-tagged SETD7 expression vectors together with a mock or a FLAG-tagged LSD1 vector. The signal intensity corresponding MYPT1 methylation was quantified by image J. C, quantitative analysis of methylated and unmethylated MYPT1 peptides analyzed by iCarta (KYA Technologies). Mean ± SD of 5 independent experiments. *P* values were calculated using Student's *t* test (**, *P* < 0.01) D, *in vitro* methyltransferase assay of wild-type N-MYPT1 (WT) and N-MYPT1 (K442A) mutant. Mean ± SD of 3 independent experiments. The *P* value was calculated using Student's *t* test (***, *P* < 0.001)

showed that methylated MYPT1 peptides were notably increased after treatment with SETD7, and sequential LSD1 treatment significantly diminished its amount. Consistently, the amount of unmethylated MYPT1 peptides was decreased after SETD7 treatment and was restored by following LSD1 treatment (Fig. 2C). Furthermore, we found that SETD7 methylated wild-type (WT)-MYPT1 in a dose-dependent manner; whereas, methylation signal of mutant MYPT1 containing a substitution of Lys 442 to alanine was much weaker (Fig. 2D), implying that Lys 442 of MYPT1 is likely to be the dominant target of methylation/demethylation dynamics regulated by SETD7 and LSD1.

Phosphorylation and dephosphorylation of RB1 is well known to be a key regulator in cell cycle progression in cancer cells (13, 14), and MP was recently reported to regulate dephosphorylation of RB1 in a MYPT1-dependent manner (11, 15, 16). Hence, we examined the relationship between MYPT1 expression and RB1 phosphorylation status. Overexpression of MYPT1 diminished RB1 phosphorylation (Ser 807/811) in 293T cells (Fig. 3A). On the other hand, the amount of phosphorylated RB1 (Ser 807/811) was increased dramatically after treatment with MYPT1 siRNAs although the level of CDK4 protein was not influenced (Fig. 3B), suggesting that the increased MYPT1 expression might induce the dephosphorylation of RB1 (Ser 807/811). We subsequently examined protein expression levels of LSD1 and MYPT1, and the RB1 phosphorylation status. After knockdown of LSD1, the increase of MYPT1 protein was observed and the amount

of phosphorylated RB1 (Ser 807/811) was decreased (Fig. 3B). In contrast, real-time PCR analysis showed that transcriptional levels of *MYPT1* and *RB1* expressions were unchanged (Supplementary Fig. S6). These data revealed that MYPT1 seems to become unstable when it is demethylated by LSD1, and decreased MYPT1 protein level might in turn increase the amount of phosphorylated RB1. So we hypothesize that MYPT1 stability may be regulated by methylation/demethylation dynamics, and the amount of MYPT1 is likely to be a key factor in the regulation of the phosphorylation status of RB1. Hence, we performed an E2F reporter assay to investigate the effect of LSD1 demethylation on the cell cycle progression. After treatment with LSD1 siRNA, E2F-luciferase activity was significantly decreased compared with that after treatment with control siRNA (Fig. 3C). To investigate biological functions of WT-MYPT1 and K442A-MYPT1, we performed an E2F reporter assay using WT-MYPT1 and K442A-MYPT1. E2F luciferase activity in 293T cells was significantly suppressed after overexpression of WT-MYPT1 compared with mock and K442A-MYPT1–transfected cells (Fig. 3D), indicating that E2F activity can be regulated by MYPT1 protein expression levels and K442 methylation is likely to be a key factor in the regulation of the phosphorylation status of RB1.

We also examined endogenous MYPT1 stability with/without exogenous LSD1 proteins after inhibiting protein synthesis by cycloheximide. Endogenous MYPT1 protein in 293T cells transfected with exogenous LSD1 degraded more rapidly
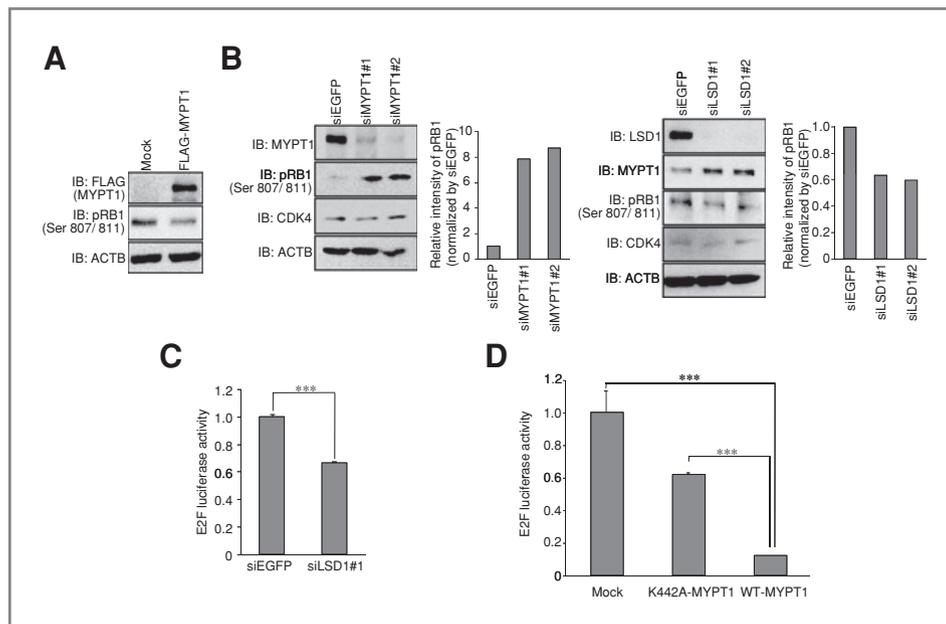
Figure 3. LSD1 regulates RB1 phosphorylation through demethylation of MYPT1. A, overexpression of MYPT1 in 293T cells. After transfection with a FLAG-tagged MYPT1 or a mock vector, Western blot analysis was done with antibodies against FLAG and RB1 (Ser 807/811). The amount of ACTB in each sample was used as an internal control. B, left; depletion of MYPT1-enhanced RB1 phosphorylation. The amount of ACTB in each sample was used as an internal control. Right; depletion of LSD1-enhanced MYPT1 expression and decreased RB1 phosphorylation (Ser 807/811). The signal intensity corresponding RB1 phosphorylation (Ser 807/811) was quantified by image J. C, E2F reporter assay after depletion of LSD1 in A549 cells. Mean $\pm$ SD of 3 independent experiments. P values were calculated using Student's t test (***, $P < 0.001$). D, E2F reporter assay after overexpression of WT-MYPT1 and K442A-MYPT1 in 293T cells. Mean $\pm$ SD of 3 independent experiments. P values were calculated using Student's t test (***, $P < 0.001$).

compared with that in mock-transfected cells (Fig. 4A). To clarify the mechanism for regulation of MYPT1 protein stability in more detail, we performed the ubiquitination assay because it was recently reported that MYPT1 stability can be regulated by the E3 ubiquitin ligase SIAH2 (17). Figure 4B illustrates that the amount of ubiquitinated MYPT1 protein in 293T cells that overexpressed full-length LSD1 was much higher than that in the cells transfected with a mock vector or in the cells with the partial LSD1 protein without the enzymatic activity (residues 1–500). In addition, after treatment with MG132, polyubiquitination of MYPT1 was observed only in the cells having the full-length LSD1 (Fig. 4C). These results suggest that overexpression of LSD1 could promote polyubiquitination of MYPT1 and destabilize MYPT1 protein in cancer cells. In addition, mutant MYPT1 containing a substitution of Lys 442 to alanine degraded more rapidly compared with WT-MYPT1 in 293T cells (Fig. 4D), indicating that Lys 442 seems to play a crucial role in the stability of MYPT1 regulated by the ubiquitin-proteasome pathway. Finally, we examined MYPT1 expression levels using $Setd7^{-/-}$ mouse embryonic fibroblast (MEF) to evaluate that methylation of MYPT1, as regulated by SETD7, can be related to the MYPT1 stability. Western blot analysis showed that MYPT1 protein expression was substantially reduced in $Setd7^{-/-}$ MEF. However, MYPT1 expression at the RNA level was not changed in $Setd7^{-/-}$ MEF cells (Supplementary Fig. S7). The data also support that the stability of MYPT1 proteins is physiologically regulated by SETD7 in vivo.

We previously reported that SMYD3, a histone methyltransferase, stimulates cell proliferation through its methyltransferase activity and plays a crucial role in human carcinogenesis (18–20). Although dysfunction of histone lysine methylation or demethylation was shown to contribute to human carcinogenesis, the mechanism how abnormal lysine methylation status of nonhistone proteins causes carcinogenesis has not been clarified. In this work we have proposed a dynamic model for the regulation of MYPT1 protein stability through lysine methylation and demethylation in cancer cells (Supplementary Fig. S8). The overexpressed LSD1 may enhance MYPT1 ubiquitination through its demethylation activity and result in the increase of the amount of phosphorylated RB1. Subsequently, released E2F activates transcription of genes required for S phase, and cell cycle progression is enhanced. Because our previous detailed cell cycle analysis using flow cytometry showed that LSD1 can be a key regulator for G_1/S transition of cancer cells (2), our findings in this study seems to be reasonable.

This is the first report showing cell cycle regulation with a mechanism on the basis of protein methylation/demethylation dynamics and consistent with these results, MYPT1 protein expression was significantly reduced in lung cancer that we previously found that LSD1 is constitutively overexpressed (ref. 2; Supplementary Fig. S9). Intriguingly, Pang and colleagues just reported that methylated proteins had significantly longer half-life than proteins for which no methylation was found on the basis of the analysis of large-scale generated peptide mass spectra (21). These data indicate that
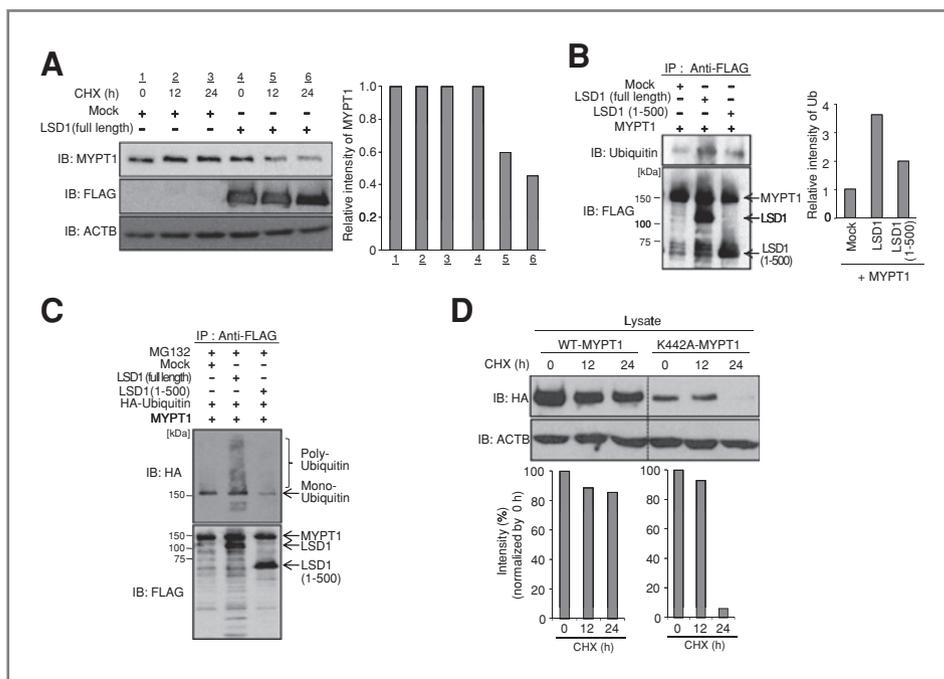
Figure 4. Protein stability of MYPT1 regulated by SETD7 and LSD1. A, LSD1 shortens the half-life of MYPT1. Cells were treated with 100 μg/mL cycloheximide (CHX) and immunoblotted to examine endogenous MYPT1 protein levels. The amount of ACTB in each sample was used as an internal control. The signal intensity corresponding MYPT1 protein was quantified by image J. B, LSD1 induced ubiquitination of MYPT1. After transfection with a FLAG-tagged MYPT1 expression vector together with a mock, a FLAG-tagged full-length LSD1 or a FLAG-tagged inactive LSD1 (1–500) expression vector, Western blot analysis was done with an antibody against ubiquitin. The signal intensity corresponding ubiquitinated MYPT1 was quantified by image J. C, LSD1-induced polyubiquitination of MYPT1. Cells were treated with 25 μmol/L MG132 for 6 hours, and FLAG-MYPT1 immunoprecipitates were prepared and immunoblotted with anti-HA (ubiquitin) and -FLAG antibodies. D, methylation of lysine 442 can be related to MYPT1 stability. Cells were treated with 100 μg/mL CHX and immunoblotted to examine exogenous MYPT1 protein levels. The amount of ACTB in each sample was used as an internal control. The signal intensity corresponding WT- and K442A MYPT1 proteins was quantified by image J.

one of the important functions of lysine methylation in nonhistone proteins can be the regulation of protein stability. Meanwhile, Wang and colleagues recently reported that LSD1 inhibits the invasion of breast cancer cells in vitro and suppresses breast cancer metastatic potential in vivo (22). As the data imply that the LSD1 may be multifunctional in cancer cells, further functional analyses will elucidate diverse functions of LSD1 in human carcinogenesis.

Recent accumulated information suggests that epigenetic changes play a crucial role in human carcinogenesis (23) and that the methylation/demethylation dynamics of histone proteins has a central role in epigenetic regulation (24). This study indicates that histone methyltransferases and demethylases seem to regulate nonhistone proteins as well, and their dysregulation can also play very critical roles in carcinogenesis.

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