BMB
Reports

BMB Rep. 2022; 55(11): 541-546
www.bmbreports.org

SET7-mediated TIP60 methylation is essential for DNA double-strand break repair

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The repair of DNA double-strand breaks (DSBs) by homologous recombination (HR) is crucial for maintaining genomic integrity and is involved in numerous fundamental biological processes. Post-translational modifications by proteins play an important role in regulating DNA repair. Here, we report that the methyltransferase SET7 regulates HR-mediated DSB repair by methylating TIP60, a histone acetyltransferase and tumor suppressor involved in gene expression and protein stability. We show that SET7 targets TIP60 for methylation at K137, which facilitates DSB repair by promoting HR and determines cell viability against DNA damage. Interestingly, TIP60 demethylation is catalyzed by LSD1, which affects HR efficiency. Taken together, our findings reveal the importance of TIP60 methylation status by SET7 and LSD1 in the DSB repair pathway. [BMB Reports 2022; 55(11): 541-546]

INTRODUCTION

DNA damage repair plays a key role in preserving genomic stability. Defeat of DNA damage repair results in an aggregation of mutagenic effects, thus increasing the risk of numerous pathological processes, including tumorigenesis. Among DNA lesions, double-strand breaks (DSBs) are produced by endogenous and exogenous DNA impairing elements, such as ionizing radiation, ultraviolet (UV) light and certain highly toxic chemicals. Non-homologous end joining (NHEJ) and homologous recombination (HR) are the two principal DSB repair pathways. Error-prone NHEJ is a DSB repair mechanism which is a DSB-end ligation response that is active all around the cell cycle. Also, NHEJ does not depend on a template. In contrast, error-free HR requires an intact template DNA. Therefore, HR only occurs during the late S/G2 phase. HR repair occurs effectively by DSB end resection with Mre11-Rad50-NBS1 (MRN) protein complex, ExoI and CtIP (1). In particular, post-translational modifications (PTMs) of non-histone proteins are essential for encouraging DNA damage repair. Many proteins associated with DNA repair systems are modulated by the control of PTMs for a fast DNA damage response (DDR). This is because unrepaired DNA leads to genome instability and causes cancer occurrence. For instance, RPA1 acetylation by PCAF is required for nucleotide excision repair, and PRMT5-dependent methylation of RUVBL1, a TIP60 coactivator, is essential for HR (2, 3). Regulation by dephosphorylation of PP4 plays a crucial role in DNA repair and cell survival (4).

Ubiquitously expressed acetyltransferase TIP60 functions in several signaling pathways such as transcriptional regulation, histone acetylation and DNA repair (5). TIP60 acetylates core histones H2A, H3, H4 and different non-histone proteins such as p53, Twist (6-8). Diverse PTMs control acetyltransferase activation of TIP60; for example, S86-phosphorylation plays an important role in modulating autophagy and apoptosis by TIP60 under various stress states (9, 10). Furthermore, TIP60 plays an important role in DSB repair by maintaining genomic stability and regulating DNA repair through its histone acetyltransferase (HAT) activity (11).

Histone H3K4-specific monomethyltransferase, SET7, is a prime methyltransferase for non-histone proteins. To date, more than 30 non-histone SET7 targets that are involved in various cellular processes, including transcriptional regulation, differentiation, and response to DNA damage, have been identified. Specially, it is reported that methylated PARP1 by SET7 is necessary for increasing enzyme activity and activating the DDR proteins (12). Therefore, SET7 is known to activate or regulate the enzymatic activity of DDR proteins and play an important role in DDR.

Our study, we discovered that SET7-mediated TIP60 is methylated at K137 in response to DNA damage. We showed that DNA damage caused by a potent DNA-damaging agent, hydroxyurea (HU), induces SET7-mediated TIP60 methylation and facilitates HR repair. Additionally, we identified that LSD1 causes the demethylation of TIP60 and regulates the DNA damage repair procedure. Finally, we revealed that methylation of TIP60, which is essential for HR-mediated DSB repair, promotes cellular proliferation in HCT116 colon cancer cells.

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https://doi.org/10.5483/BMBRep.2022.55.11.080

Received 3 May 2022, Revised 23 May 2022, Accepted 5 July 2022

Keywords: DNA repair, Epigenetics, Methylation, SET7, TIP60

ISSN: 1976-670X (electronic edition)
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RESULTS

SET7 methylates TIP60 at Lys 137 *in vitro* and *in vivo*

TIP60 is an acetyltransferase that plays a role in DNA repair and apoptosis by acetylating histones. Moreover, it plays an important role in DDR signal induction (11). To further investigate the post-translational modification of TIP60 in the DNA repair process, we determined TIP60 methylation by SET7. We selected SET7 among the methyltransferases because it is known for a major methyltransferase for diverse non-histone proteins, and we also reported that SET7-mediated UHRF1 methylation is required for DSB repair (13). First, we performed an *in vitro* methylation assay by incubating recombinant GST-TIP60 and GST-SET7. Interestingly, TIP60 was methylated by SET7 in a dose-dependent manner; however, the catalytic null mutant SET7 H297A did not methylate TIP60 (Fig. 1A). To determine whether SET7 methylated TIP60 *in vivo*, we tested the methylation level of TIP60 via an IP assay with anti-methyl lysine antibodies. An increase in TIP60 methylation levels was observed in HCT116 cells overexpressing SET7 (Fig. 1B). When

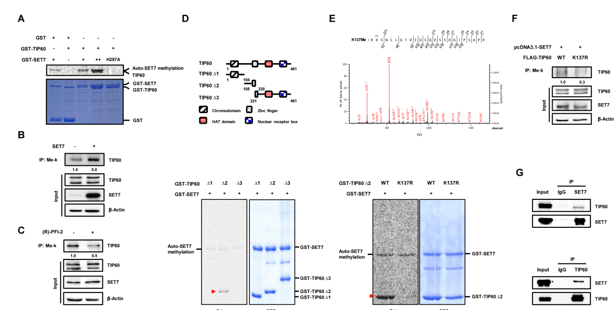

Fig. 1. SET7/9 methylates TIP60 *in vitro* and *in vivo*. (A) Purified GST-TIP60 was incubated overnight at 30°C with increasing amounts of GST-SET7. Reaction mixtures were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and stained by Coomassie or exposed to an autoradiographic film. (B) SET7 was overexpressed in HCT116 cells. IPs using anti-methyl lysine antibody were performed. Methyl lysine levels were normalized by input of TIP60. (C) HCT116 cells were treated with 1 uM SET7 inhibitor (R)-PFI-2 for 24 h. IPs using an anti-methyl lysine antibody were performed. Methyl lysine levels were normalized by input of TIP60. (D) Purified GST-TIP60 deletion mutants were incubated overnight at 30°C with GST-SET7. Reaction mixtures were separated by SDS-PAGE and analyzed via a phosphorimager. (E) Mass spectrometry analysis (LC-MS/MS) was performed, and methylation status of the K137 residue was determined. Purified GST-TIP60Δ2 or GST-TIP60Δ2 point mutants were incubated overnight at 30°C with GST-SET7. Reaction mixtures were separated by SDS-PAGE and analyzed via a phosphorimager. (F) HEK293T cells were transfected with the indicated plasmids and IPs using an anti-methyl lysine antibody were performed. Methyl lysine levels were normalized by input of TIP60. (G) pcDNA3.1-SET7 and Flag-TIP60 were overexpressed in HCT116 cells. The cell lysates were immunoprecipitated with an anti-SET7 antibody. Associated proteins were eluted, resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and immunoblotted using the indicated antibodies.

SET7 was ectopically overexpressed in SET7 knockdown cells, the methylation level of TIP60 was increased; in contrast, SET7 H297A did not rescue the methylation level of TIP60 (Supplementary Fig. 1A). It is known that the methyltransferase activity of SET7 is inhibited by (R)-PFI-2 (14). When cells were treated with (R)-PFI-2, TIP60 methylation was significantly inhibited in HCT116 cells (Fig. 1C). To accurately investigate the methylation site of TIP60, we performed an *in vitro* methylation assay using TIP60 deletion constructs TIP60 Δ1, Δ2, and Δ3. Among the deletion constructs tested, only TIP60 Δ2 was methylated by SET7 (Fig. 1D). To accurately identify the major methylation sites of TIP60, LC-MS/MS was performed on a high-resolution orbitrap instrument after *in vitro* methylation assay, using recombinant SET7 and TIP60. As a result of mass spectrometry, the methylated lysine residue of TIP60 was K137 (Fig. 1E upper panel). An *in vitro* methylation assay was performed with SET7 and TIP60 Δ2 mutants in which K137 was replaced with arginine (TIP60 Δ2 K137R) to confirm the results of the LC-MS/MS analysis. Compared with TIP60 Δ2, the methylation of TIP60 in TIP60 Δ2 K137R was not detected (Fig. 1E lower panel). Interestingly, TIP60 K137 is an evolutionarily conserved residue across various species, indicating the functional importance of this residue (Supplementary Fig. 1B). This indicates that the major methylation site of TIP60 is K137. When we compared the methylation levels between TIP60 WT and TIP60 K137R *in vivo*, the methylation level of TIP60 K137R was lower than that of TIP60 WT (Fig. 1F). Altogether, our data demonstrate that SET7 methylates K137 of TIP60 both *in vitro* and *in vivo*. Next, we examined the interaction between TIP60 and SET7. Formerly, we confirmed that TIP60 methylation by SET7 did not affect TIP60 expression levels or TIP60-mediated acetylation (Supplementary Fig. 2A, B). TIP60 is mainly localized in the nucleus (6). Therefore, we analyzed the cellular fraction to confirm that SET7 did not affect TIP60 localization (Supplementary Fig. 2C). Co-IP assays were performed in pcDNA3.1-SET7 or Flag-TIP60 overexpressed cells, and the results indicated that interaction between SET7 and TIP60 *in vivo* (Fig. 1G). To confirm the interaction between TIP60 and SET7 *in vitro*, we performed a GST pull-down assay using TIP60 constructs (Supplementary Fig. 3A). Next, we conducted a co-IP assay in Flag-empty vector (EV) or Flag-TIP60 and pcDNA3.1-SET7 overexpressed cells to demonstrate the interaction between SET7 and TIP60 in cells, and the results indicated that TIP60 interacts with SET7 *in vivo* (Supplementary Fig. 3B). Together, these data suggest that SET7 interacts with TIP60 both *in vitro* and *in vivo*.

HU-mediated DNA damage induces TIP60 methylation and promotes HR for DSB repair

HU is a replication inhibitor that causes DSB by depleting the nucleotide pool and causing replication fork arrest (15). We set the experimental condition for HU treatment at 5 mM HU concentration 4 hours because we identified that the apoptosis was induced enough in this condition (Supplementary Fig. 4A).

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To investigate whether TIP60 methylation is important for DNA damage signals, we confirmed the TIP60 methylation level after treating HCT116 cells with HU. The TIP60 methylation level was significantly increased by HU-induced DNA damage (Fig. 2A). However, HU did not induce TIP60 methylation when SET7 was depleted (Supplementary Fig. 4B). An IP assay was performed with and without HU to examine whether the interaction between TIP60 and SET7 was affected by HU-induced DNA damage. Remarkably, the interaction between TIP60 and SET7 was significantly increased in response to DNA damage by HU (Supplementary Fig. 4C).

HU, which is primarily dynamic in the S phase of the cell cycle, induces HR in mammalian cells by inhibiting replication (16, 17). Additionally, HR and SET7 are associated with DDR by catalyzing the methylation of DDR protein ARTD1, and HR occurs during the S and G2 phases of the cell cycle (12, 18). Therefore, we focused on the influence of TIP60 methylation by SET7 on the HR repair mechanism.

TIP60 methylation levels were confirmed at each stage of the cell cycle to determine the relationship between TIP60 methylation and its role in HR. In Fig. 2B, the results of increased TIP60 methylation levels in the S phase suggest that TIP60 methylation can play an important role in HR. These results showed that methylation of TIP60 is induced by SET7 in response to DNA damage. Accordingly, we performed an integrated reporter assay to determine whether TIP60 methylation affects HR efficiency (Fig. 2C upper panel). Since TIP60 methylation by SET7 induces the HR process, we tested whether methylation-deficient TIP60 K137R could promote HR. The HR reporter assay demonstrated that TIP60 WT promoted HR in SET7 overexpressed cells, but not in TIP60 K137R expressed cells (Fig. 2C lower panel). In addition, when we overexpressed SET7 WT and SET7 H297A (catalytic mutant) in HCT116 cells and measured HR efficiency, the result showed that TIP60 methylation by SET7 promoted HR (Supplementary Fig. 4D). To determine the effect of TIP60 on RPA and Rad51 foci formation on DNA damage sites, HU-treated shTIP60 cells were subjected to an immunofluorescence staining assay. In contrast to the effects of TIP60 WT, RPA foci was blocked in cells transfected with TIP60 K137R (Fig. 2D). We also identified Rad51 foci in TIP60 WT (Supplementary Fig. 4E).

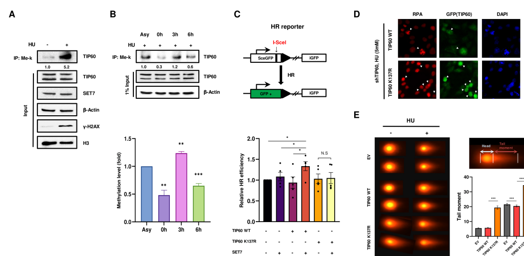

Fig. 2. Hydroxyurea (HU)-mediated DNA damage induces SET7-dependent methylation of TIP60 and induces homologous recombination (HR). (A) HCT116 cells were treated with 5 mM HU for 4 h. IPs using an anti-methyl lysine antibody were performed. Methyl lysine levels were normalized by input of TIP60. (B) HCT116 cells were arrested at the G1/S checkpoint by double thymidine block/release, and the cells were then treated with 5 mM HU for 4 h. IPs using an anti-methyl lysine antibody were performed. Methyl lysine levels were normalized by input of TIP60. Results were shown as the mean ± SEM; n = 3, $***P < 0.001$, $**P < 0.01$. (C) Schematic diagram of the HR reporter. PcDNA3.1-SET7, Flag-TIP60 WT, or Flag-TIP60 K137R was subjected to HR assay. Results were shown as the mean ± SEM; n = 5, $*P < 0.05$, N.S: no significant difference. (D) HCT116 cells with TIP60 knockdown were transfected with TIP60 WT or TIP60 K137R. RPA foci was examined following 5 mM HU treatment for 4 h. (E) HCT116 shTIP60 cells overexpressing SET7 were transfected with the indicated plasmid. Then, cells were treated with HU and subjected to a neutral comet assay. The tail moment was determined using CaspLab software, and 90 individual comets were counted for each sample (lower panel). Results were shown as the mean ± SEM; n = 90, $***P < 0.001$.

Next, we performed a neutral comet assay using shTIP60 cells to determine whether TIP60 methylation by HU promotes DNA repair. The length of the comet tail moment indicates the extent of DNA breakage. In TIP60 K137R cells, the comet tail moment increased significantly after HU treatment, whereas in TIP60 WT cells, the tail moment was shorter. Also, when compared to the control, the comet tail moment of TIP60 K137R cells was increased more than that of TIP60 WT after HU treatment (Fig. 2E). These data indicate that the degree of DNA breaks in TIP60 WT cells was significantly lower than that in TIP60 K137R cells. Taken together, our data suggest that HU increases SET7-mediated TIP60 methylation and this promotes HR in DNA-damaged cells.

LSD1 mediates demethylation of TIP60

SET7 can methylate histone H3K4, and LSD1 is the major H3K4 demethylase (19, 20). Studies have shown that LSD1 demethylates SET7-mediated methylated proteins, including UHRF1 and DNMT (13, 21). Before investigating if LSD1 can catalyze the demethylation of TIP60, we performed an IP assay to check the interaction between LSD1 and TIP60. The results indicated that endogenous TIP60 interacted with LSD1 in HCT116 cells (Fig. 3A). Next, we discovered that the TIP60 methylation level was increased in cells treated with the LSD1 inhibitor, GSK-LSD1 (Fig. 3B), suggesting that LSD1 is accountable for the demethylation of TIP60. To further test this, we overexpressed empty vector or Flag-LSD1 in LSD1 knockdown HCT116 cells and performed IP assays with an anti-methyl lysine antibodies. The TIP60 methylation level was significantly increased in LSD1 knockdown cells, whereas the methylation level was decreased in cells rescued by LSD1 overexpression (Supplementary Fig. 5). Since we identified that TIP60 methylation was damage-dependent in this study, we performed an IP assay to determine whether demethylation of TIP60 by LSD1 is also damage-dependent. We found that the increased level of TIP60 methylation in HU-treated cells was decreased when LSD1 was overexpressed (Fig. 3C). Additionally, we demonstrated

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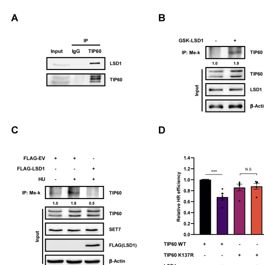

Fig. 3. LSD1 demethylates TIP60 methylation. (A) Cell extracts from HCT116 cells were immunoprecipitated with anti-TIP60 antibodies. Immunoprecipitates were eluted, resolved by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and immunoblotted with the indicated antibodies. (B) HCT116 cells were treated with 500 nM GSK-LSD1 for 24 h. IPs using an anti-methyl lysine antibody were performed. Methyl lysine levels were normalized by input of TIP60. (C) HCT116 cells were transfected with Flag-EV of Flag-LSD1 and treated with 5 mM HU for 4 h. IPs using an anti-methyl lysine antibody were performed. Methyl lysine levels were normalized by input of TIP60. (D) Flag-LSD1, Flag-TIP60 WT, or Flag-TIP60 K137R were subjected to the homologous recombination (HR) assay. Results were shown as the mean ± SEM; n = 5, ***P < 0.001, N.S: no significant difference.

the effect of LSD1-induced TIP60 demethylation on HR by confirming HR efficiency using the HR reporter assay. LSD1 reduced HR efficiency with TIP60 WT, but LSD1 did not affect HR efficiency in TIP60 K137R-transfected cells (Fig. 3D). Collectively, these results indicated that methylation of TIP60 is modulated by SET7 and LSD1 in the DNA repair procedure.

SET7-mediated TIP60 methylation promotes colon cancer cell proliferation

Substances such as HU, thymidine, and camptothecin induce replication fork collapse and strongly induce HR in mammalian cells. These substances require HR for cell survival (17). UHRF1 methylation by SET7 is necessary for cell survival in response to UV exposure (13). Therefore, we investigated whether SET7-mediated TIP60 methylation regulates cell proliferation and apoptosis upon HU treatment by flow cytometry. It was confirmed that TIP60 K137R increased apoptosis in a HU-dependent manner compared to TIP60 WT (Supplementary Fig. 6A, Fig. 4A upper panel). We also investigated apoptosis through the expression level of cleaved caspase-3, an apoptosis marker (Fig. 4A lower panel). Additionally, we measured apoptosis in shLSD1 cells by treating HU. In cells overexpressing TIP60 in shLSD1, there was no change in apoptosis before and after HU was treated, and in control cells, the number of apoptotic cells increased compared to shLSD1 when HU was treated (Supplementary Fig. 6B). Next, we observed the viability of cells with TIP60 WT and methylation-deficient mutant overexpression treated with 5 mM HU for 4 h. We performed a colony formation assay. The number of colonies increased in TIP60 WT cells. However, we observed a decrease in the number of colonies in the methylation-deficient mutant (TIP60 K137R)-treated cells (Fig. 4B). To further investigate these observations, we performed a MTT assay. TIP60 knockdown cells transfected with TIP60 WT showed higher cell proliferation compared to TIP60 knockdown cells. In contrast, cells transfected with the methylation-deficient mutant (TIP60 K137R) did not show a significant increase in cell proliferation compared to the TIP60 knockdown cells (Fig. 4C). TIP60 knockdown cells showed increased proliferation compared to that of control cells, which was in agreement with the finding that overexpression of TIP60 reduced HCT116 proliferation (22). In addition, we performed MTT assay with LSD1 inhibitor, GSK-LSD1. Control cells showed increased cell proliferation when treated with GSK-LSD1 and similar results were obtained in TIP60 overexpressing cells (Supplementary Fig. 6C). Altogether, these results suggest that SET7-dependent methylation of TIP60 promotes cancer cell proliferation.

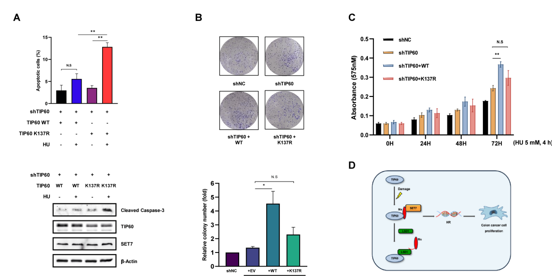

Fig. 4. TIP60 methylation by SET7 promotes cell proliferation. (A) HCT116 cells were transfected with the indicated plasmids and treated with 5 mM HU for 4 h. Apoptotic cells were measured by fluorescence-activated cell sorting (FACS) analysis, and protein expression was confirmed through a western blot assay. Results were shown as the mean ± SEM; n = 3, **P < 0.01, N.S: no significant difference. (B) Representative colony formation assay was performed using HCT116 cells with TIP60 knockdown transfected with TIP60 WT or TIP60 K137R. Cells were treated with 5 mM HU for 4 h and incubated in fresh media for 7 days. Results were shown as the mean ± SEM; n = 3, *P < 0.05, N.S: no significant difference. (C) Cell viability was determined using the MTT assay. HCT116 cells with TIP60 knockdown were transfected with TIP60 WT or TIP60 K137R. Cells were treated with 5 mM HU for 4 h and incubated in fresh media for 0-72 h. Results were shown as the mean ± SEM; n = 3, **P < 0.01, N.S: no significant difference. (D) Schematic showing how SET7-mediated TIP60 methylation promotes the homologous recombination (HR)-mediated DNA repair pathway.

DISCUSSION

Our study presents a new perspective on TIP60 function in the DNA repair process by PTMs. We showed that SET7 methylates TIP60 and promotes the HR-mediated DSB repair process. Specifically, our current study suggests that TIP60 methylation, which is significantly increased in the S phase, effectively promotes HR. Abnormal expression of TIP60 has been reported in prostate and colon cancer (22, 23). However, decreased levels

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of nuclear TIP60 have been reported in breast cancer, which demonstrates that TIP60 functions to shelter cells from genomic insecurity (24). Studies suggest that TIP60 acetylates ataxia-telangiectasia mutated (ATM) and induces autophosphorylation to activate DDR in cancer cells. Furthermore, TIP60-mediated DNA damage repair is essential for the maintenance of kidney cells (25). These results, along with those of our current study, suggest that TIP60 is not only involved in the maintenance of differentiation but also the proliferation of cells via the regulation of the DDR signaling process.

SET7 plays multiple roles in DDR by catalyzing the methylation of a series of non-histone proteins such as p53, E2F1, and SIRT1 (26, 27). Additionally, we have identified that SET7-mediated UHRF1 methylation in response to DNA damage, polyubiquitinates PCNA, and promotes HR progression (13). We also reported that PARP1 is required for the recruitment of methylated UHRF1 to DNA damage sites and for the HR repair pathway (28). Several studies have indicated that SET7 is also involved in the regulation of PARP1 activity. To further elucidate the mechanism by which TIP60 methylation by SET7 induces the HR repair pathway, it is necessary to investigate PARP1 as a mediator that recruits and interacts with TIP60 in damaged lesions in more detail. In addition, SET7 is predominantly in the cytosol and TIP60 is located in nucleus, as indicated by Supplementary Fig. 2C. However, further research is needed on where and how TIP60 goes when it is methylated by SET7.

A recent study suggested that the histone demethylase LSD1 is recruited to DNA damage sites, where it is involved in DSB repair via interaction with the E3 ubiquitin ligase RNF168 (29). We also reported that SET7-mediated UHRF1 methylation in response to DNA damage and that LSD1 reduces UHRF1 methylation, further blocking HR progression, suggesting an interesting role of the SET7-LSD1 axis in DDR signaling (13). Since LSD1 demethylates SET7-mediated methylated proteins and plays both roles in DDR, additional research is needed on the relationship between SET7 and LSD1 on TIP60 during DNA damage.

Overall, we found that TIP60 is methylated by SET7 and that this SET7-dependent methylation of TIP60 induced by DNA damage is essential for the HR repair pathway. Furthermore, we showed that TIP60 methylation by SET7 promoted cell proliferation and the potential relationship between TIP60 methylation and the HR repair pathway (Fig. 4D). Our study, we present a new mechanism of DNA damage repair based on the methylation status of TIP60 by SET7 and LSD1.

MATERIALS AND METHODS

Materials and methods are available in the supplemental material.

ACKNOWLEDGEMENTS

This research was supported by the National Research Foundation of Korea (NRF) grant from the Ministry of Science, ICT & Future Planning [NRF-2021R1A2C1013553] and was supported by the Chung-Ang University Research Scholarship Grants in 2020.

CONFLICTS OF INTEREST

The authors have no conflicting interests.

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