The methyltransferase SETD6 regulates Mitotic progression through PLK1 methylation

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Lysine methylation, catalyzed by protein lysine methyltransferases (PKMTs), is a key player in regulating intracellular signaling pathways. However, the role of PKMTs and the methylation of nonhistone proteins during the cell cycle are largely unexplored. In a recent proteomic screen, we identified that the PKMT SETD6 methylates PLK1—a key regulator of mitosis and highly expressed in tumor cells. In this study, we provide evidence that SETD6 is involved in cell cycle regulation. SETD6-deficient cells were observed to progress faster through the different mitotic steps towards the cytokinesis stage. Mechanistically, we found that during mitosis SETD6 binds and methylates PLK1 on two lysine residues: K209 and K413. Lack of methylation of these two residues results in increased kinase activity of PLK1, leading to accelerated mitosis and faster cellular proliferation, similarly to SETD6-deficient cells. Taken together, our findings reveal a role for SETD6 in regulating mitotic progression, suggesting a pathway through which SETD6 methylation activity contributes to normal mitotic pace.

Significance

The involvement of nonhistone protein methylation in cellular essential pathways is a rising field. Here we show evidence for the involvement of direct lysine methylation of the mitosis regulator PLK1 by SETD6 methyltransferase in cell cycle progression. Our results reveal that this methylation occurs on two lysine residues and that lack of methylation leads to enhanced PLK1 catalytic activity, causing accelerated mitosis pace and thus faster proliferation rates. These findings suggest that PLK1 methylation by SETD6 controls the pace of mitotic progression, greatly enhancing our understanding of cell cycle complexity. The role of methylation in mitotic progression raises the possibility of its involvement in tumorigenic pathways by orchestrating cell division rates and might have insightful implications for the development of cancer-specific markers.

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of identifying new therapeutic targets and developing alternative treatment strategies for cancer.

**Results**

**SETD6 Binds and Methylates the Mitosis Regulator PLK1 in Vitro and in Cells.** In a recent proteomic screen, in which more than 9,500 proteins were tested, we identified 118 novel SETD6 substrates, of which more than 25% are associated with oncogenic signaling pathways (21). One of the identified substrates was the Polo-like kinase 1 (PLK1) protein (Fig. 1A). PLK1 is known as a master regulator of mitosis and is involved at numerous steps during mitosis, including the G2/M checkpoint, mitotic entry, spindle formation, APC activation, and cytokinesis (4). To validate the methylation of PLK1 by SETD6, we performed an in vitro methylation assay in the presence of purified recombinant GST-SETD6 and His-PLK1, using H-labeled S-adenosyl methionine as the methyl donor. As shown in Fig. 1B, we found that His-PLK1 is methylated by SETD6. In vitro methylation was further supported by an ELISA showing that recombinant PLK1 directly interacts with SETD6 (Fig. 1C). MBP-RelA served as our positive control for the reaction (15). Methylation of endogenous PLK1 by SETD6 was detected in MDA-MB-231 cells using the MBT pull-down approach, which serves as an affinity reagent that specifically binds lysine-methylated proteins (22). Methylation of PLK1 was abolished upon SETD6 silencing, or when the MBTD355N mutant was used for the pull-down (Fig. 1D). These findings indicate that SETD6 binds and methylates PLK1 in vitro and in cells.

**Depletion of SETD6 Results in Faster Progression Through Mitotic Steps.** Methylation of PLK1 by SETD6 led us to hypothesize that SETD6 might contribute to regulation of the cell division process. To examine the involvement of SETD6 in different steps of the cell cycle, SETD6 knockout (KO) HeLa cells were generated using the CRISPR-Cas9 system (SI Appendix, Fig. S1A). To study the involvement of SETD6 in cell cycle progression, control and SETD6 KO HeLa cells were synchronized to GAP 1 phase/synthesis phase (G1/S) using the double-thymidine block method. At different time points after release, cells were fixed and stained using propidium iodide (PI) to determine DNA content, after which they were subjected to FACS analysis. At 1 h after thymidine release, ~80% of all cells were in the S phase (SI Appendix, Fig. S1B), which correlated with previous findings (23). The time course of the cell cycle of these cells revealed that mitotic exit and G1 entry occurred faster in the two SETD6 KO cells compared with the control cells, as shown at 9 and 10 h post-thymidine release (Fig. 2 and SI Appendix, Fig. S1B). Early exit from mitosis could be explained by shorter mitosis time; however, mitosis is divided into several stages, each characterized by a distinctive morphology. To elucidate the mitotic step in which SETD6 has the greatest impact, synchronized cells were fixed and stained with antitubulin antibody, which serves as a marker for the different stages in the cell cycle (24), as well as with DAPI stain. The percentage of cells at different stages of mitosis (Fig. 2B) (prometaphase, metaphase, anaphase, and cytokinesis) was quantified according to their typical morphology (as shown in the representative images) at different times post-thymidine release (Fig. 2B). In the prometaphase, no significant difference between control and KO cells was observed. However, 8 h after thymidine release, the number of cells in the metaphase, the anaphase, and cytokinesis was higher in the SETD6-depleted cells. Interestingly, 10 h after thymidine release, the percentage of control cells in cytokinesis peaked whereas the SETD6 KO cells were already decreasing. These results strongly indicate that SETD6-deficient cells progress through mitosis faster and that this phenomenon is observed during different steps of mitosis.

**SETD6 Silencing Promotes Accelerated Proliferation.** To support these findings in unsynchronized cells, we performed live cell imaging in control and SETD6-depleted HeLa cells. mCherry-H2B and GFP-tubulin were coexpressed in the cells to determine mitotic progression. Since the prophase is considered the mitosis initiation point, it was determined as t = 0; entry into the proceeding steps was counted from this point. Images from two representative movies are shown in Fig. 3A. The SETD6 KO cells were shown to progress along all mitotic phases at faster rates (Fig. 3A and Movies S1 and S2). Importantly, no differences in morphology of the cells were noted (Fig. 3A). Accelerated mitosis in SETD6-depleted cells suggests that cells proliferate faster upon SETD6 silencing. To test this hypothesis, we compared the proliferation rate of synchronized control and SETD6 KO cells over 120 h after release from thymidine block. An increase in the proliferation rate between the cell lines was first seen after ~72 h, which represents three full cell cycles, with a greater effect observed at 120 h (Fig. 3C and SI Appendix, Fig. S2A showing two additional and independent SETD6 KO cells). This indicates that depletion of SETD6 leads to increased cellular proliferation. To test if this increased proliferation is SETD6 dependent, we generated SETD6 KO cells stably expressing SETD6 KO cells stably expressing SETD6 (SI Appendix, Fig. S2B). Re-expression of SETD6 in the SETD6 KO cells resulted in a partial rescue of the accelerated proliferation phenotype (Fig. 3C), confirming that SETD6 contributes to the observed phenomena.

**SETD6-Depleted Cells Show Greater PLK1 Catalytic Activity.** Structural analysis reveals that PLK1 is composed of a common N-terminal catalytic domain, a C-terminal regulatory domain with highly conserved sequences named the Polo box domain (PBD), and an interdomain linker (25, 26). Studies have shown that the activation of PLK1 can be regulated by phosphorylation at Thr-210 (25, 26). When inactive, the PBD binds to the catalytic domain, which inhibits access of substrates to the catalytic domain. PLK1 phosphorylation at Thr-210 induces dissociation of the...
PBD from the catalytic domain, increasing kinase activity (4, 25, 26). PLK1 undergoes phosphorylation at Thr-210 during mitosis and cytokinesis, which may regulate its function in these processes (4), and thus serves as an indication of PLK1 catalytic activity (27). Our observations that SETD6 specifically binds and methylates PLK1 and that depletion of SETD6 correlates with accelerated mitosis suggested that these phenomena are related to PLK1 kinase activity. To test this possibility, we performed quantitative immunofluorescence in unsynchronized control and SETD6 KO cells, using a phospho-specific PLK1 antibody for Thr-210. We detected a significant increase in phosphorylation on this site in SETD6-depleted cells, during both the prometaphase and metaphase of mitosis.

![Image of flow cytometry data showing cell cycle phases](image1.jpg)

**Fig. 2.** SETD6-depleted cells show faster mitotic progression. (A) HeLa control cells and two SETD6 KO cell lines were synchronized using double-thymidine block. After release, cells were fixed at indicated time points and stained with PI to differentiate cell cycle phases using flow cytometry. (Top) Histogram of PI staining in control and two SETD6 KO cells showing cells released from mitosis into G1 phase at 9 and 10 h after thymidine release. The presented experiment represents three experiments in which similar results were obtained. In each experiment, data were obtained from 50,000 single cells for each condition. (Bottom) Quantification of cells released from mitosis into G1 phase at 9 h (Left) and cells remaining in G2/M at 10 h (Right) after thymidine release. Results were averaged from three independent experiments, and statistical analysis was performed with the standard t test. *P < 0.05. (B) HeLa control and SETD6 KO cells were synchronized using double-thymidine block. After release, cells were fixed at indicated times and stained for tubulin and DAPI to determine different cell cycle phases. Three to five different images were captured from each condition, and three independent experiments were performed (each dot represents an image). Cells in the indicated mitotic steps were manually counted. Representative images of steps are shown. Statistical analysis was performed using the unpaired, two-tailed t test. ns, not significant. *P < 0.01, **P < 0.001, ***P < 0.0001.

![Image of immunofluorescence images](image2.jpg)

**Fig. 3.** SETD6 silencing promotes accelerated mitosis proliferation. (A) Live cell imaging of wt and SETD6 KO cells transfected with GFP-tubulin and mCherry-H2B. Z slices were acquired at 10-min intervals using a confocal spinning-disk microscope. Shown are maximal intensity projections. Time 0 indicates the first frame where the prophase was detected. Frames taken from representative videos are shown. (Scale bars, 10 μm.) See also Movies S1 and S2. (B) Average duration of each indicated mitotic step was calculated from n = 25 for each condition. Statistical analysis was performed using the unpaired, two-tailed t test. ***P < 0.0001. (C) Control and SETD6 KO cells stably expressing an empty vector or SETD6 were synchronized using double-thymidine block. After release, cells were counted every 24 h as indicated, using PrestoBlue reagent. Each measurement was normalized to the t(0) value taken right after release from thymidine. SD represents quadruplicates of a representative experiment out of three, all showing similar results. Statistical analysis was performed using the unpaired, two-tailed t test. **P < 0.001, ***P < 0.0001.
and the metaphase (Fig. 4 A and B), suggesting that SETD6 depletion primes an increase in the enzymatic activity of PLK1. To validate this notion, we examined the specific PLK1 phosphorylation of the PBD-binding protein (PBIP1). PLK1 was previously shown to specifically phosphorylate the centromeric protein PBIP1 at Thr78, a phosphorylation event that engages a docking site for a high-affinity interaction between the PBD of PLK1 and PBIP1-pT78 (27, 28). We used a phospho-specific antibody to quantify PBIP1-pT78 fluorescence intensity in the presence and absence of SETD6 (Fig. 4 C and D). A considerable rise in phosphorylated PBIP1 staining upon SETD6 silencing was noted, reflecting the elevation of PLK1 kinase activity. Consistent with these results, FACS analysis of unsynchronized control and SETD6 KO HeLa cells revealed an increase in PLK1-pT210 and PBIP1-pT78 levels during G2/M in SETD6-depleted cells (Fig. 4E and SI Appendix, Fig. S3). Altogether, the presented data suggest that diminishing SETD6 in cells correlates with the enhancement of PLK1 catalytic activity, followed by accelerated mitosis.

**SETD6 Binds and Methylates PLK1 During Mitosis.** Having demonstrated that SETD6 binds and methylates PLK1 together with the observation that SETD6 regulates mitosis progression, we next tested whether these two phenomena are linked. To this end, we synchronized control HeLa cells and followed the endogenous expression of both SETD6 and PLK1 after release from thymidine block and their ability to interact with one another. We found that SETD6 protein levels, like those of PLK1, peak during mitosis (9 h after release) and that the physical interaction between the proteins is better detected at this stage (SI Appendix, Fig. S4A). To test whether methylation is carried out at this point, lysates of control and SETD6 KO cells were collected 9 h after block release and immunoprecipitated using a pan-methyl antibody. A clear reduction in the PLK1 methylation signal was observed in the SETD6 KO cells (SI Appendix, Fig. S4B). The residual signal of methylated PLK1 in the absence of SETD6 suggests that, in addition to SETD6, PLK1 may be subjected to methylation by one or more other enzymes. Taken together, these findings demonstrate that, 9 h after synchronization, a time frame in which cells are in the mitotic phase, SETD6 binds and methylates PLK1.

**SETD6 Methylates PLK1 at Lysines 209 and 413.** To specifically map the site of PLK1 methylation by SETD6, recombinant His-PLK1 was incubated under methylation reaction conditions with recombinant His-SETD6 followed by mass spectrometry (MS) analysis (Fig. 5A). The MS identified two potential methylation sites (Fig. 5A): Lys-209, which resides in the kinase domain, adjacent to the functionally essential Thr-210 residue, and Lys-413, which is located at Polo box 1, next to the tryptophan residue at position 414, also known to be critical for phospho-substrate binding and centromeral localization (29). To validate the MS analysis, we generated the K209R and K413R single mutants as well as the double K209R/K413R mutant and tested them using in vitro methylation reaction to determine if the purified mutants are methylated by His-SETD6. As shown in Fig. 5B, the methylation of PLK1 K209R and K413R mutants was reduced compared with the wild type (wt). Notably, we found a greater reduction in methylation of the double mutant, implying that both residues are methylated by SETD6 (Fig. 5B). We next examined whether SETD6 methylates PLK1 at K209 and K413 in cells. To this end, lysates of HeLa SETD6 KO cells, transfected with Flag-PLK1 wt or the double mutant K209R/K413R, in the presence or absence of HA-SETD6 were immunoprecipitated using a pan-methyl antibody followed by Western blot analysis (Fig. 5C). Consistent with our previous findings (Fig. 1 and SI Appendix, Fig. S4B), PLK1 wt was methylated by SETD6. As predicted, PLK1 was methylated to a lesser extent when the double mutant was used for transfection (Fig. 5C and SI Appendix, Fig. S5 for quantification), indicating that K209 and K413 are the primary target lysines for methylation by SETD6 also in cells.

**PLK1 Methylation at Lys-209 and Lys-413 by SETD6 is Required for Its Timely Controlled Catalytic Activity.** Mapping PLK1 methylation sites enabled us to examine the physiological relevance of this

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**Fig. 4.** PLK1 kinase activity increases during mitosis upon SETD6 silencing. Control and SETD6 KO cells were stained for DNA, tubulin, and PLK1-pT210 (A and B) or PBIP1-pT78 (C and D), as indicated. (A and C) Representative images for each antibody are shown. (Scale bar, 10 μm.) (B and D) Quantification of fluorescence intensity of cellular PLK1-pT210 (B) or PBIP1-pT78 (D) staining in control and SETD6 KO HeLa cells at prometaphase and metaphase (n ≥ 12 cells per condition). (E) FACS analysis of control and SETD6 KO HeLa cells stained with PLK1-pT210 or PBIP1-pT78 antibodies. PI staining was used to gate cells at G2/M. Averages represent three independent experiments performed in duplicates, with data acquired from 25,000 single cells in each experiment. Statistical analysis was performed using the unpaired, two-tailed t test. *P < 0.01, **P < 0.001, ***P < 0.0001.
Fig. 5. SETD6 methylates PLK1 on two lysine residues: K209 and K413. (A) MS/MS spectra showing monomethylation of PLK1 at Lys-209 and Lys-413. Recombinant PLK1 was digested from a gel after overnight methylation reaction with recombinant SETD6, followed by MS analysis. (Bottom) Schematic diagram of PLK1 domain structures: kinase domain, destruction box (D box) domain, and the two Polo box domains (PB1 and PB2). The localization of the two SETD6-methylated lysine residues is indicated, as well as the Thr-210 and W414 residues, which are essential for PLK1 activity. (B) In vitro methylation assay in the presence of S-hydroxymethyl-5-adenosylmethionine with recombinant His-PLK1 wt and the indicated mutants and His-SETD6. (C) Coomassie stain of the recombinant proteins used in the reactions. (C) Lysates from HeLa SETD6 KO cells transfected with Flag-PLK1 wt or Flag-PLK1 K209R/K413R with or without HA-SETD6 were immunoprecipitated with pan-methyl antibodies and blotted with Flag antibodies. (Bottom) Levels of Flag-PLK1, HA-SETD6, and GAPDH (loading control) in the total extracts.

methylation event in mitosis and proliferation regulation. Consistent with the elevation in the proliferation rate of SETD6-depleted cells (Fig. 5C), synchronized HeLa control cells stably expressing the Flag-PLK1 K209R/413R mutant (SI Appendix, Fig. S6A) demonstrated a higher proliferation rate compared with cells expressing the PLK1 wt, although to a lesser extent than the HeLa SETD6 KO cells (Fig. 6A), indicating that SETD6 might be involved in mitotic regulation through additional pathways. Similarly to the SETD6-depleted cells, the increased proliferation rate in cells expressing the PLK1 K209R/413R mutant can be explained by an acceleration in mitotic progression (SI Appendix, Fig. S6B). Fluorescence quantification of PLK1-pT210 and PBIP1-pT78 revealed an increase in unsynchronized control cells expressing the Flag-PLK1 K209R/413R mutant compared with Flag-PLK1 wt (Fig. 6 B and C and SI Appendix, Fig. S7). In agreement with these findings, PLK1-pT210 and PBIP1-pT78 levels were observed to increase following the introduction of Flag-PLK1 K209R/413R and re-expression of SETD6 into SETD6-deficient cells, in comparison with Flag-PLK1 wt (Fig. 6 D and E and SI Appendix, Fig. S8). These findings correlate with our observations of SETD6-depleted cells (Figs. 2–4), supporting our conclusion that direct methylation of PLK1 by SETD6 regulates mitotic pace, by tuning PLK1 activity.

Discussion

Many reports have implicated histone methylation in cell cycle maintenance and progression (30). However, little is known about the role of methylation of nonhistone proteins in these processes. In this study, we showed that methylation of a non-histone protein by the PKMT SETD6 regulates cell cycle progression. In a previous proteomic screen, we identified the cell cycle regulator PLK1 as a potential SETD6 substrate. We demonstrated that SETD6 directly binds and methylates PLK1 in vitro and in cells on two lysine residues (K209 and K413) during mitosis. Lack of methylation leads to acceleration of all mitotic steps, eventually leading to early mitotic exit (Fig. 6F). One might suspect such mitotic perturbation to yield mitotic defects, such as improper chromosome segregation and alignment, spindle assembly deficiencies, and many others. However, no visual morphological changes were noted in cells lacking PLK1 methylation by SETD6. This suggests that SETD6 methylation has a moderate impact on each mitotic step, which, on one hand, has a great effect on the overall mitotic rate but, on the other hand, seems not to interfere with different mitotic checkpoints in charge of preventing cell division abnormalities.

The lysine at position 209 is adjacent to the threonine residue at position 210, the phosphorylation of which is required for PLK1 proteolytic processing in each mitotic step, with or without methylation by SETD6. In agreement with our conclusion that direct methylation of PLK1 by SETD6 regulates mitotic pace, by tuning PLK1 activity.

**SI Appendix**
activation (31). The second methylated lysine, positioned at 413, is adjacent to another highly important residue—tryptophan 414—which is located in the first Polo box and is critical for substrate recognition through nonpolar interactions (32). The strategic positions of the two methylated lysines and their relevance to PLK1 enzymatic activity led us to suspect that SETD6 is physiologically involved in this feature, which is crucial during the course of mitosis. The local activation of PLK1 by SETD6 showed a remarkable elevation of the phosphorylated state of both PLK1 itself and one of its most established substrates, PBIP1. These findings strongly indicate that SETD6 methylation is required for fine-tuning of PLK1 kinase activity, thus dictating the pace of mitosis. Having said that, our working model does not exclude the possibility that additional players participate in this regulatory network, such as proteins that are involved in the activation of PLK1, which might also be regulated directly or indirectly by methylation, or additional methyltransferases besides SETD6 that methylate PLK1 and regulate its binding and catalytic activity. We therefore propose a significant role for SETD6 cellular activity in controlling mitosis progression and exit, mediated by PLK1 methylation. Given the high complexity of cell cycle regulation, it is believed that additional mechanisms participate in the fine-tuning of this essential process. Nevertheless, in the complex cell cycle regulatory network, in which many proteins function in a redundant and overlapping context to compensate for and amend any disruption, such a notable effect reveals a key player in the regulation of mitosis maturation. Understanding the dynamic regulation of the cell cycle is crucial not only in normal physiological conditions but also in pathological states such as aberrant cell proliferation during cancer initiation and development. Many studies have demonstrated a key role for PLK1 regulation in mitosis, which contributes to the onset of tumorigenesis as well as the poor prognosis of cancer (33). PLK1 is therefore considered both a biomarker and a target for highly specific cancer therapy (34). Indeed, recent advances in the development of PLK1 inhibitors for cancer management have been published (35).

The involvement of SETD6 in cancer regulation has yet to be revealed; however, our data point out a possible link between SETD6 cellular activity and cancer progression through methylation of PLK1 and cell cycle regulation. Thus, understanding the contribution of lysine methylation in general and SETD6 in particular to cell cycle fine regulation promises progress toward identifying cancer-specific markers and therapeutic targets.

Methods
More details are provided in the SI Appendix.

Flow Cytometry. For cell cycle analysis without intracellular staining, cells were harvested at different times after thymidine release, fixed using 70% ethanol, stained using PI in the presence of RNaseA for 20 min, and then analyzed using the BD FACSAria system (BD Biosciences). For intracellular staining, unsynchronized cells were fixed using 4% paraformaldehyde (PFA), permeabilized using 90% methanol and stained with the relevant primary antibodies, followed by fluorochrome-conjugated secondary antibodies. Then cells were stained with PI in the presence of RNaseA for 20 min and analyzed using the Guava easyCyte flow cytometer (Merck). Data obtained were analyzed using FlowJo analysis software (TreeStar).

Live Cell Recording. Cells were plated at low density in 4-well chamber slides (Ibidi), transfected with GFP-tubulin and mCherry-H2B, and imaged 24 h later. Z stacks of cells were collected for 12 h at 10-min intervals using a fully confocal confocal spinning-disk microscope (Marianas; Intelligent Imaging) with 40x oil objective (numerical aperture, 1.3), and video-recorded on an EMCCD camera (Evolve; Photometrics).

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