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 toward 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.

PLK1 | SETD6 | lysine methylation | cell cycle | mitosis

The cell division cycle is commonly divided into two major phases: interphase and mitosis (M phase). During interphase, cells accumulate nutrients, grow, and duplicate their DNA. During the M phase, the cells separate their DNA into two sets (mitosis) and then undergo cytokinesis, during which the cytoplasm of the cells splits in two to form two identical daughter cells. All steps of the cycle are tightly regulated at multiple levels, including gene expression, protein-protein interaction, protein stability, and posttranslational modifications (1). One key regulator of the cell cycle—and especially mitosis—is the protein Polo-like kinase 1 (PLK1), which is overexpressed in many cancers (2). Intensive investigation during the last decades demonstrated that PLK1 kinase activity is essential in numerous stages of mitosis, including functional maturation of centrosomes and bipolar spindle assembly, M phase entry, nuclear envelope breakdown, sister chromatid cohesion and formation of kinetochore-microtubule attachments, mitotic exit, and cytokinesis (3). PLK1 regulation of the different cell cycle phases is mediated by the recruitment and phosphorylation of several substrates, among them PBIP1, BubR1, Cdc25C, CCNB1, and many others (4). Although PLK1 activity is initially present at GAP 2 phase (G2), when it is known to promote mitotic entry after a checkpoint-dependent arrest (5), PLK1 activity reaches peak levels during mitosis (6).

The kinase activity of PLK1 throughout the cell cycle is precisely orchestrated by the modulation of its stability, localization, conformation, and catalytic activity using posttranslation modifications (PTMs), including phosphorylation (5), dephosphorylation (7), ubiquitination (8), and recently shown sumoylation (9). During mitosis, PLK1 is phosphorylated by the Aurora A and B kinases on a threonine residue (Thr-210) positioned in its activation loop (T-loop), and this phosphorylation is required for its activity (5, 10). PLK1 consists of an N-terminal kinase domain and a C-terminal Polo box domain (PBD), which binds to prime-phosphorylated substrates. PLK1 kinase activity is regulated by its unique conformation; the PBD interacts with the kinase domain and suppresses its kinase activity when Thr-210 is not phosphorylated in interphase cells. This inhibitory interaction is absent in mitotic PLK1, which is phosphorylated on Thr-210 (11, 12). Active PLK1 is required for mitosis entry and progression and the initiation of cytokinesis, after which it is degraded (13).

Lysine methylation, catalyzed by protein lysine methyltransferases (PKMTs), is a PTM that plays a key role in a variety of essential cellular signaling pathways (14). In recent years, the PKMT SETD6 has been shown to participate in the NF-κB cascade (15, 16), the nuclear factor erythroid 2–related factor 2 (NRF2) oxidative stress response (17), the Wnt signaling pathway (18), nuclear hormone receptor signaling (19), and embryonic stem cell differentiation (20), all of which are vital cellular processes.

Here we identify a role for SETD6 in regulating cell cycle progression. We provide biochemical and cellular evidence that SETD6 specifically binds and methylates PLK1 during mitosis, and we map the methylation site to two lysine residues located at positions 209 and 413. We further show that depletion of SETD6, as well as the double substitution of the lysine residues (K209/413R), leads to elevation in PLK1 catalytic activity. This enzymatic rise results in the acceleration of the different mitotic steps, ending with early cytokinesis. This phenomenon is reflected on the cellular level by an enhanced proliferation rate, a phenotype which is partially rescued by stable re-expression of SETD6. We believe that these findings have broad implications for both basic and translational research and hold the potential

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 promotion. 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. 1*A*). 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 ^{3}H-labeled S-adenosyl methionine as the methyl donor. As shown in Fig. 1*B*, 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. 1*C*). 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. 1*D*). 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. S1*A*). 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 propridium 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. S1*B*), 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*A* and *SI Appendix*, Fig. S1*B*). 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. 2*B*) (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. 2*B*). 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.

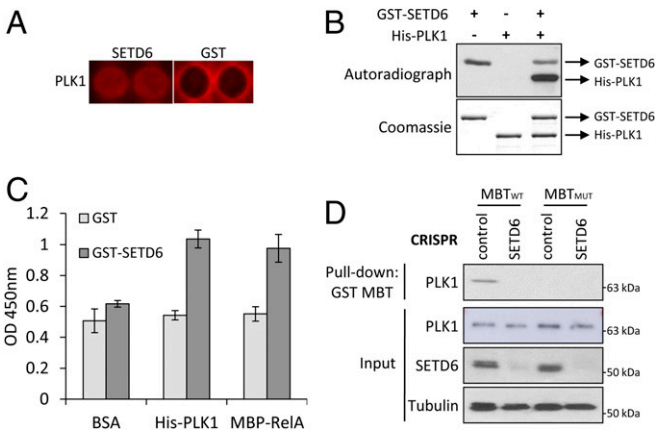

Fig. 1. SETD6 binds and methylates PLK1 in vitro and in cells. (*A*) Positive methylation signal of recombinant PLK1 by GST-SETD6 shown in a protoarray experiment (21). Protoarrays were incubated overnight under PKMT reaction conditions with GST-SETD6 and GST as negative control. Arrays were then probed with pan-methyl antibodies followed by fluorophore-conjugated secondary antibodies. (*B*) In vitro methylation assay in the presence of ^{3}H-labeled S-adenosyl methionine with recombinant His-PLK1 and GST-SETD6, incubated together overnight. Coomassie stains of the recombinant proteins used in the reactions are shown. (*C*) ELISA-based analysis of the interaction between recombinant GST-SETD6 and His-PLK1. The 96-well plate was coated with 2 μg of His-PLK1 or BSA as negative control or MBP-RelA as positive control, and then covered with 0.5 μg of GST-SETD6 or GST. Signal detection was achieved using anti-GST antibodies followed by secondary HRP-conjugated antibodies (error bars, SEM). (*D*) Methylated proteins were pulled down from cellular extracts of MDA-MB-231 control and SETD6 KO cells with GST-MBT to detect methylation differences on endogenous PLK1. As negative control, pull-down was also performed with the MBTD355N mutant.

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. 3*A*. The SETD6 KO cells were shown to move along all mitotic steps at a faster pace (Fig. 3 *A* and *B* and Movies S1 and S2). Importantly, no differences in morphology of the cells were noted (Fig. 3*A*). 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. 3*C* and *SI Appendix*, Fig. S2*A* 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 (*SI Appendix*, Fig. S2*B*). Re-expression of SETD6 in the SETD6 KO cells resulted in a partial rescue of the accelerated proliferation phenotype (Fig. 3*C*), 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

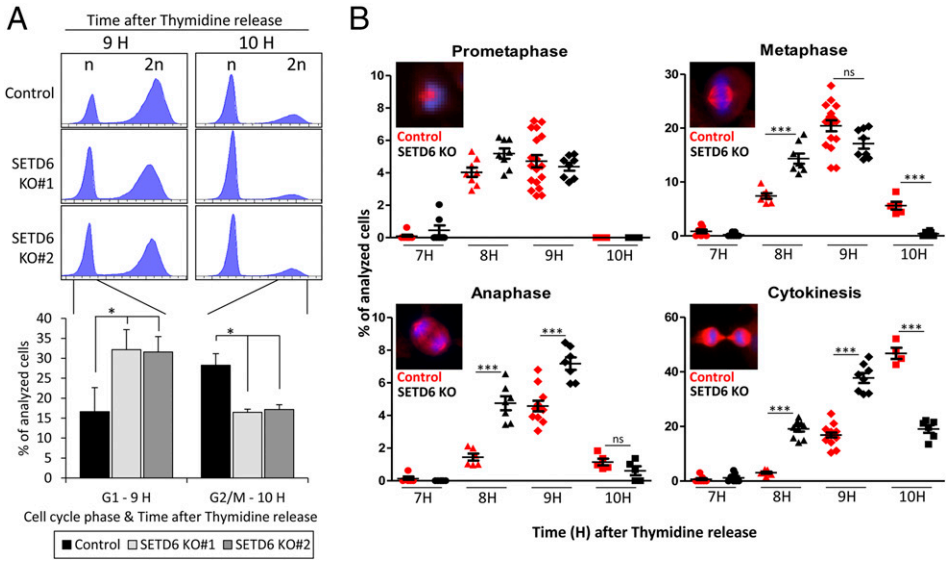

Fig. 2. SETD6-depleted cells show faster mitotic progression. (*A*) HeLa control cells and two SETD6 KO cell lines were synchronized using double-thymidine block. After release, cells were fixed at indicated time points and stained with PI to differentiate cell cycle phases using flow cytometry. (*Top*) 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. $*P < 0.01$, $**P < 0.001$, $***P < 0.0001$.

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

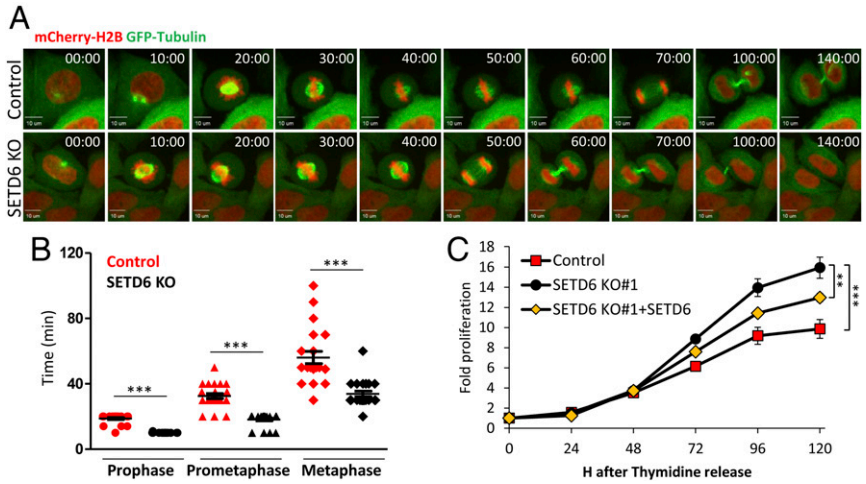

Fig. 3. SETD6 silencing promotes accelerated mitosis proliferation. (*A*) Live cell imaging of wt and SETD6 KO cells transfected with GFP-tubulin and mCherry-H2B. Z slices were acquired at 10-min intervals using a confocal spinning-disk microscope. Shown are maximal intensity projections. Time 0 indicates 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 generates 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. 4*E* 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. S4*A*). 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. S4*B*). 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. 5*A*). The MS identified two potential methylation sites (Fig. 5*A*): 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 centrosomal 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. 5*B*, 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. 5*B*). 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. 5*C*). Consistent with our previous findings (Fig. 1 and *SI Appendix*, Fig. S4*B*), PLK1 wt was methylated by SETD6. As predicted, PLK1 was methylated to a lesser extent when the double mutant was used for transfection (Fig. 5*C* 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

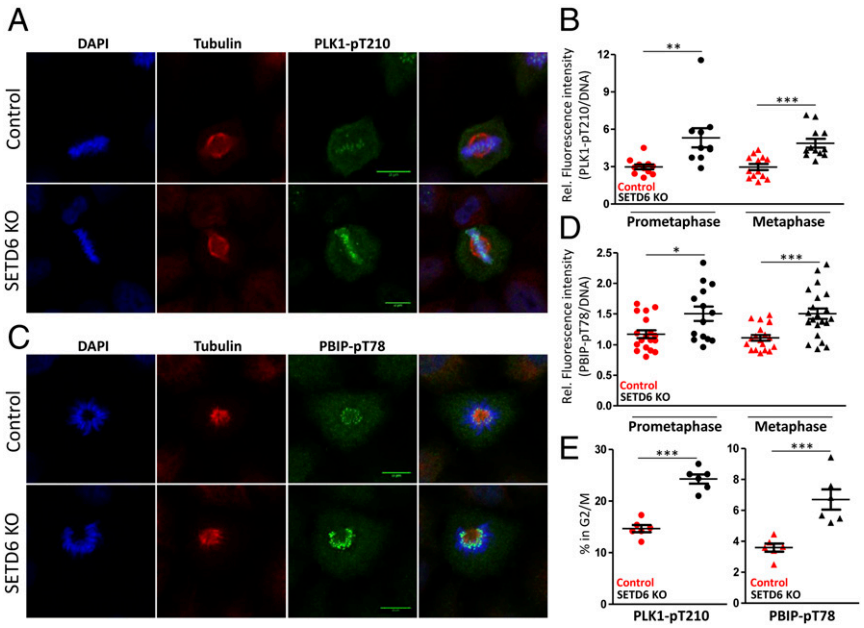

Fig. 4. PLK1 kinase activity increases during mitosis upon SETD6 silencing. Control and SETD6 KO cells were stained for DNA, tubulin, and PLK1-pT210 (*A* and *B*) or PBIP1-pT78 (*C* and *D*), as indicated. (*A* and *C*) Representative images for each antibody are shown. (Scale bar, 10 μm.) (*B* and *D*) 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 ($n \geq 12$ 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. $*P < 0.01$, $**P < 0.001$, $***P < 0.0001$.

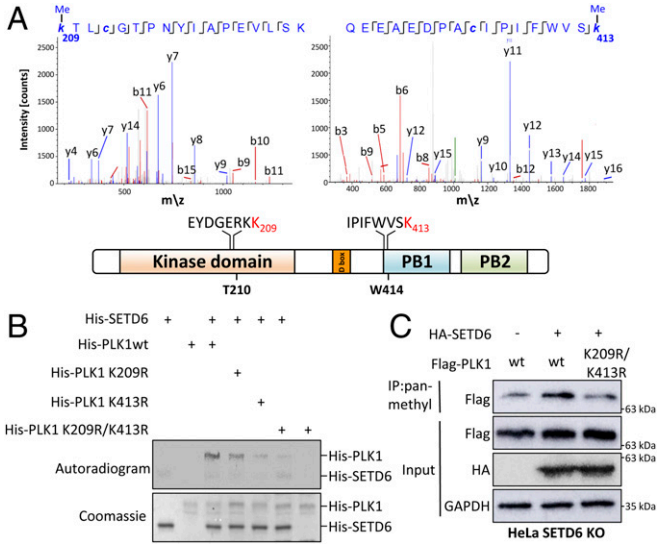

Fig. 5. SETD6 methylates PLK1 on two lysine residues: K209 and K413. (*A*) MS/MS spectra showing monomethylation of PLK1 at Lys-209 and Lys-413. Recombinant PLK1 was digested from a gel after overnight methylation reaction with recombinant SETD6, followed by MS analysis. (*Bottom*) Schematic diagram 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 ^{3}H-labeled S-adenosyl methionine with recombinant His-PLK1 wt and the indicated mutants and His-SETD6. (*Bottom*) 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 using 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. 3*C*), synchronized HeLa control cells stably expressing the Flag-PLK1 K209R/413R mutant (*SI Appendix*, Fig. S6*A*) demonstrated a higher proliferation rate compared with cells expressing the PLK1 wt, although to a lesser extent than the HeLa SETD6 KO cells (Fig. 6*A*), 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. S6*B*). 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 nonhistone 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. 6*F*). 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

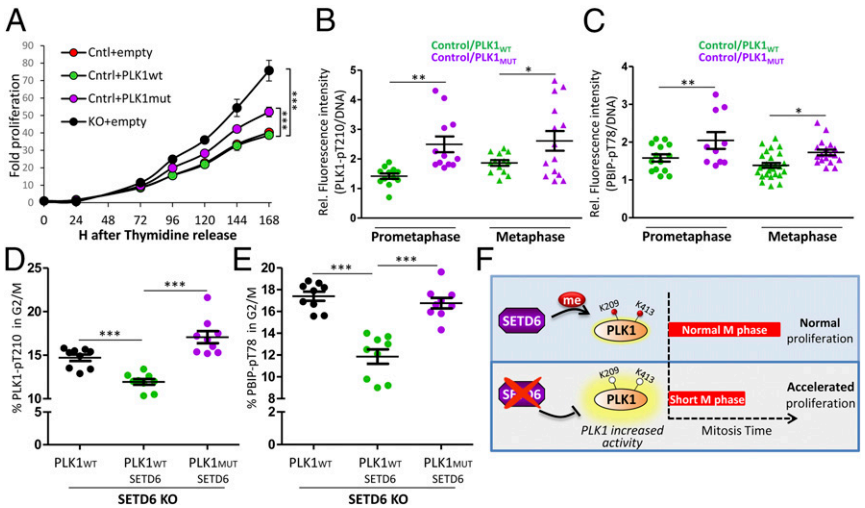

Fig. 6. K209R/K413R double substitution leads to accelerated mitosis and increased PLK1 catalytic activity. (*A*) HeLa control cells stably expressing either empty plasmid, Flag-PLK1wt, or Flag-PLK1 K209R/K413R and SETD6 KO cells were synchronized using double-thymidine block. After release, cells were counted every 24 h using PrestoBlue reagent. Each measurement was normalized to the *t*(0) value taken right after release from thymidine block. SD represents quadruplicates of a representative experiment out of two, all showing similar results. Statistical analysis was performed using the unpaired, two-tailed *t* test. ***$P < 0.0001$. (*B* and *C*) Control HeLa cells overexpressing Flag-PLK1 wt or Flag-PLK1 K209R/K413R mutant were stained for DNA, tubulin, and PLK1-pT210 (*B*) or PBIP1-pT78 (*C*), as indicated. Quantification of the fluorescence intensity of the indicated antibody staining in control and SETD6 KO HeLa cells at prometaphase and metaphase was obtained from $n \geq 14$ cells per condition. (*D* and *E*) HeLa SETD6 KO cells transfected with Flag-PLK1 wt or Flag-PLK1 K209R/K413R with or without HA-SETD6 were stained using PLK1-pT210 (*D*) or PBIP1-pT78 (*E*) antibodies and subjected to FACS analysis. PI staining was used to gate cells at G2/M. Averages represent three independent experiments performed in triplicates, with data obtained from 25,000 single cells in each experiment. Statistical analysis was performed using the unpaired, two-tailed *t* test. *$P < 0.01$, **$P < 0.001$, ***$P < 0.0001$. (*F*) Schematic model of the effect of SETD6 on mitotic progression. When SETD6 is absent, PLK1 is not methylated on K209 and K413; its kinase activity arises, leading to increased mitotic pace and resulting in accelerated proliferation.

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. Indeed, lack of methylation 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 methyltrasnferases 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 conjunction and redundancy 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 fluorophore-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 data 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 incubated confocal spinning-disk microscope (Marianas; Intelligent Imaging) with 40× oil objective (numerical aperture, 1.3), and video-recorded on an EMCCD camera (Evolve; Photometrics).

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