Polo-like Kinase 1-mediated Phosphorylation of Forkhead Box Protein M1b Antagonizes Its SUMOylation and Facilitates Its Mitotic Function*

Jinglei Zhang, Chengfu Yuan, Jianguo Wu, Zeinab Elsayed, and Zhengan Fu

From the Department of Human and Molecular Genetics, Virginia Commonwealth University Institute of Molecular Genetics, Virginia Commonwealth University Massey Cancer Center, Virginia Commonwealth University School of Medicine, Richmond, Virginia 23298

**Background:** PLK1-mediated phosphorylation of FoxM1b leads to full activation of FoxM1b.

**Results:** FoxM1b SUMOylation leads to suppression of FoxM1b activity, which is abrogated by PLK1-mediated phosphorylation.

**Conclusion:** PLK1-mediated phosphorylation of FoxM1b antagonizes its SUMOylation and, thereby, facilitates its mitotic function.

**Significance:** Elucidating the PLK1-mediated regulation of FoxM1b would greatly facilitate targeted therapeutic interventions.

Transcription factor Forkhead box protein M1b (FoxM1b) plays an important role during mitotic entry and progression. Our previous studies identified polo-like kinase 1 (PLK1) as a major regulator of FoxM1b. During G2/M transition, PLK1 directly interacts with and phosphorylates FoxM1b, resulting in full activation of the transactivation capacity of FoxM1b. Such a vital regulatory mechanism is essential for timely mitotic entry and progression. However, the molecular mechanism by which PLK1-mediated phosphorylation enhances the transcriptional activity of FoxM1b remains to be determined. We demonstrate that FoxM1b can be SUMOylated in vitro and in vivo, preferentially by SUMO-1. SUMOylation of FoxM1b was found to occur at multiple sites, leading to suppression of FoxM1b transcriptional activity. Such a posttranslational modification of FoxM1b was antagonized by PLK1-mediated phosphorylation. By immunofluorescence staining and subcellular fractionation, we demonstrate that SUMO conjugation promotes cytosolic translocation of FoxM1b. Moreover, SUMO modification of FoxM1b facilitates the ubiquitin-mediated proteasomal degradation of FoxM1b. PLK1-mediated phosphorylation of FoxM1b abrogates the inhibitory effect on FoxM1b by SUMO modification, thereby promoting its nuclear translocation and preventing its proteolytic degradation in the cytoplasm. Such an antagonistic regulatory mechanism is essential for the mitotic function of FoxM1b, ensuring timely mitotic entry and progression. Taken together, our studies have revealed a working mechanism by which PLK1 positively regulates the activity and level of FoxM1b, which would greatly facilitate therapeutic interventions that focus on targeting the PLK1-mediated and/or FoxM1b-mediated signaling network.

Forkhead box protein M1 (FoxM1) is in the Fox family of transcription factors whose members have a conserved forkhead DNA-binding domain (1). FoxM1 is important for cell differentiation, proliferation, cellular transformation, angiogenesis, apoptosis, and metastasis (2). As a key mitotic player, FoxM1 regulates mitotic entry and progression by controlling the expression of a cluster of G2/M target genes, including Cyclin B1, Cdc25B, Survivin, CENPA, and Aurora B (3, 4). Depletion of FoxM1 results in severe defects in mitosis and cytokinesis (5). FoxM1-null mice die during embryogenesis because of the development of polyploid cardiomyocytes and hepatocytes. FoxM1-deficient (−/−) mouse embryonic fibroblasts display chromosomal instability and polyploidy, leading to cell death. Abnormal activation of FoxM1 is a hallmark of many human cancers (6). FoxM1 expression increases with tumor grade and is inversely correlated with patient survival (2). FoxM1 overexpression promotes anchorage-independent growth and tumor formation in nude mice (7), indicating that it contributes to cellular transformation and tumorigenicity.

The human FoxM1 gene is mapped to chromosome 12p13-3, which consists of ten exons, two of which (exons A1 and A2) are spliced differentially, giving rise to three distinct isoforms (splice variants): FoxM1a, FoxM1b, and FoxM1c (8–10). FoxM1a has been found to be transcriptionally inactive because of the disruption of the transactivation domain by exon A2. Its physiological function has not been investigated (10). In contrast, most studies to date have focused on FoxM1b (containing neither the A1 nor the A2 exon) and FoxM1c (harboring only exon A1) (8, 10). These two isoforms are transcriptionally active and can directly transactivate target gene expression in an isoform-specific manner (11–13). The functional difference and interrelationship among the three human FoxM1 isoforms remain to be determined.

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1 Both authors contributed equally to this work.

2 To whom correspondence should be addressed: Dept. of Human and Molecular Genetics, Virginia Commonwealth University School of Medicine, 401 College St., Richmond, VA. Tel.: 804-628-3843; Fax: 804-628-2054; E-mail: zfu@vcu.edu.
FoxM1 is a typical proliferation-associated transcription factor (14). It is ubiquitously expressed in proliferating cells and is barely detectable in quiescent, senescent, or terminally differentiated cells (8, 15). Both the expression level and transcriptional activity of FoxM1 vary throughout the cell cycle. They are low in G₀ and G₁ phases, begin to rise at the onset of S phase, and peak at G₂/M phase (8), which correlates positively with the critical roles of FoxM1 in proliferation and mitosis. As a key transcription factor that mediates diverse cellular processes, FoxM1 is under highly coordinated and multilayered regulation. It has been shown that not only mRNA and protein expression of FoxM1 but also its transcriptional activity are positively and negatively regulated by proliferation and anti-proliferation signals, respectively (14).

Posttranslational modifications play an important role in the regulation of FoxM1. For instance, phosphorylation of FoxM1 by Raf-MEK-ERK is responsible for its nuclear translocation in late S phase (16, 17). Hyperphosphorylation during G₂/M phase correlates with increased transcriptional activity of FoxM1, suggesting that phosphorylation plays an important regulatory role in fully activating this protein during the late phases of the cell cycle (16). Using yeast two-hybrid screening, we previously identified FoxM1b as a novel PLK1-interacting protein (18). During G₂/M transition, PLK1 directly interacts with and phosphorylates FoxM1b, activating its transcriptional activity (18). PLK1-mediated FoxM1b regulation controls a large array of G₂/M target genes that are required for timely mitotic entry and progression (18). This study provides the working mechanisms of how FoxM1 is activated in the later phases of the cell cycle and how it contributes to G₂/M progression. Moreover, these important findings establish a novel link between PLK1 and a key mitotic transcription factor, FoxM1b, thereby providing some clues as to how PLK1 globally regulates cell division. However, the precise mechanism by which PLK1-mediated phosphorylation enhances the transcriptional activity FoxM1 remains to be determined.

In this study, we investigated how PLK1-mediated phosphorylation of FoxM1b results in increased transcriptional activity of FoxM1b. We show that PLK1-dependent phosphorylation antagonizes the SUMO³ modification of FoxM1b, thereby leading to its nuclear retention and protection from proteasomal degradation during G₂/M progression. Therefore, our results suggest that PLK1 activates FoxM1b transcriptional activity by restricting SUMO conjugation to FoxM1b.

**EXPERIMENTAL PROCEDURES**

**Plasmids, shRNA, and Chemicals—**pA3M-Myc-FoxM1b (WT, EE (a phosphomimetic mutant), and AA (an unphosphorylatable mutant) of FoxM1b), the reporter 6× FoxM1-TATA-luciferase reporter construct, pRES-FLAG-CMV-PLK1 (WT, constitutively active (TD), and catalytically inactive (KD) mutants), and pGEX-FoxM1b have been described previously (18). pCMV-Ubc9 was provided by W. T. Beck (University of Illinois). The MT107-His-Ub expression vector was a gift from Dr. D. Bohmann (University of Rochester Medical School).

Plasmids for amino acid substitution mutants of pA3M-Myc-FoxM1b (Lys-201, Lys-218, Lys-341, Lys-445, Lys-463, and Lys-480 were converted to Arg) and pA3M-Myc-FoxM1b-AA/6KR were generated by PCR-based mutagenesis (Stratagene). cDNA encoding SUMO-1 was amplified by PCR and subcloned in-frame to the 5’end of the WT FoxM1b cDNA to generate pA3M-Myc-SUMO-1-FoxM1b. The sequences of primers were as follows: forward, aaaaacggtatgtcgacagaggccaa; reverse, aaaaacgggtatcagctccatccctggtttctgtgaataac. Lentivirus-based plasmids expressing shRNA to target FoxM1b (pLOK.1-FoxM1b), mimosine, thymidine, aphidicolin, blebbistatin, nocodazole, and cycloheximide were purchased from Sigma.

**Cell Culture, Transfection, and Synchronization—**HeLa, HeLa S3, and 293T cells were grown in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 mg/ml streptomycin. U2OS cells were grown in DMEM (Invitrogen) containing 10% fetal bovine serum, 100 units/ml penicillin, and 100 mg/ml streptomycin. HeLa cells stably expressing hexahistidine-tagged SUMO-1 were provided by R. T. Ray (University of Dundee). Transient transfection of U2OS, HeLa, and 293T cells was performed with Lipofectamine 2000 according to the instructions of the manufacturer. To establish stable U2OS-derived cell lines, transfected cells were diluted and grown in media containing 800 µg/ml G418 for 14 days. Individual clones were then selected and maintained in media containing 400 µg/ml G418. For G₁ phase arrest, cells were treated with 0.5 mM mimosine for 24 h. For S phase arrest, cells were treated with 4 mM hydroxyurea for 40 h. For G₂/M arrest, cells were treated with 100 ng/ml nocodazole for 16 h. To synchronize HeLa and U2OS cells at the G₁/S boundary, cells were treated with 2 mM thymidine for 18 h, released for 10 h, and then treated with 2 µg/ml aphidicolin for 18 h. It was a challenge to synchronize cells at anaphase and telophase because of the short duration of anaphase. Because the non-muscle myosin II ATPase motor provides the force for furrow ingression, treatment with the myosin II inhibitor blebbistatin leads to cytokinesis failure (20). Matsui et al. (19) reported that treatment with blebbistatin at a low concentration (50 µM) slows down cleavage furrow ingression and extends the duration of anaphase, allowing synchronization of cell populations in anaphase and telophase. The following protocol has been reported previously to successfully enrich cell populations in different stages of the mitosis and was used in this study (19). HeLa S3 cells were treated with 4 mM thymidine for 24 h, released into thymidine-free medium for 9 h, and then incubated with nocodazole for 4 h. Prometaphase cells were collected by mitotic shakeoff. These cells were washed to remove nocodazole and released in nocodazole-free medium for 20 min to enrich metaphase cells. Subsequently, the cells were incubated in the presence of 50 µM blebbistatin for 20 and 50 min to enrich cells in anaphase and telophase, respectively.

**Antibodies, Immunoblotting, and Immunoprecipitation—**

The antibodies used in this study were as follows: anti-Cyclin B1 (1:1000), anti-FoxM1 (1:200), and anti-Myc (1:500) (Santa Cruz Biotechnology); anti-SUMO-1 (1:1000, University of Iowa); anti-Aurora B (1:250) and anti-Cdc25B (1:250) (BD Biosciences); anti-Ubiquitin (1:1000, Upstate); anti-Lamin A/C (1:1000) (Santa Cruz Biotechnology); anti-PLK1 (1:500) (Santa Cruz Biotechnology); anti-α-tubulin (1:1000, Sigma).
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(Cell Signaling Technology); and anti-FLAG (1:5000), anti-β-actin (1:5000), anti-α-tubulin (1:5000), and anti-SUMO-2/3 (1:1000) (Sigma). Rabbit polyclonal antibodies recognizing the phosphorylated serine 715 (anti-p715) of FoxM1b have been described previously (18). Cells were lysed in NETN lysis buffer (150 mM NaCl, 1 mM EDTA, 20 mM Tris (pH 8), 0.5% Nonidet P-40) containing protease and phosphatase inhibitors for 20 min at 4 °C. Cell lysates were centrifuged for 10 min at 4 °C. For immunoprecipitation or communoprecipitation experiments, cleared lysates (1–2 mg of protein) were immunoprecipitated with the indicated polyclonal antibodies and protein A-Sepharose for 1 h. Proteins were separated on SDS-PAGE, transferred to Immobilon P membranes, and immunoblotted with the indicated antibodies.

Subcellular Protein Fractionation—Cells were lysed in hypotonic buffer (10 mM HEPES-KOH, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT, 0.2 mM PEFA 1023 (pH 7.9), and 0.5% Nonidet P-40) and then centrifuged for 10 s at 16,000 × g at 4 °C. The supernatants were collected as cytoplasmic extracts. The pellets were washed twice with hypotonic buffer and lysed with high-salt buffer (450 mM NaCl, 1 mM PMSF, 50 mM Tris (pH 7.4), 0.2 mM Na₃VO₄, 5 mM β-glycerophosphate, 20% glycerol, 2 mM DTT, and 1% Nonidet P-40). Cell lysates were centrifuged for 15 min at 16,000 × g at 4 °C, and the supernatants were collected as nuclear extracts. Equal amounts of protein from cytoplasmic and nuclear extracts were then analyzed by Western blotting.

Luciferase Assays—U2OS and HeLa cells were transfected with the indicated plasmids using Lipofectamine 2000 according to the instructions of the manufacturer. Luciferase activity was determined 24 h after transfection using the Dual-Luciferase reporter assay system (Promega). Luciferase levels were normalized to Renilla luciferase activity. The promoter activity resulting from transfection with FoxM1b WT was set at 100%, and relative luciferase activity is shown. Experiments were performed in triplicate, and statistical analysis was performed using Microsoft Excel.

Immunostaining—Cells grown on coverslips were fixed for 15 min with 3% paraformaldehyde solution and then permeabilized with 0.5% Triton X-100 for 5 min. After washing with PBS three times, the slides were incubated with primary antibodies for 20 min at 37 °C, and then fluorescein isothiocyanate-conjugated secondary antibody (Jackson ImmunoResearch Laboratories) for 20 min at 37 °C. All antibodies were diluted in 5% goat serum. Cells were counterstained with DAPI dye (EMD Millipore) for 30 s.

In Vitro SUMOylation Assay—GST-FoxM1b was expressed in the Escherichia coli BL21 strain as reported previously (18). The SUMOylation reaction was carried out using an in vitro SUMOylation kit from Active Motif according to the instructions of the manufacturer. After termination with SDS-PAGE sample buffer, reaction products were separated on SDS-PAGE and Western blotted with anti-SUMO-1 and anti-FoxM1 antibody.

Cell Cycle Analysis by Flow Cytometry—Cells were harvested, washed with PBS, and fixed with ice-cold 70% ethanol for at least 1 h. Cells were washed twice in PBS and treated for 30 min at 37 °C with RNase A at 5 μg/ml and propidium iodide at 50 μg/ml. The cells were then analyzed on a FACSscan flow cytometer (BD Biosciences). The percentage of cells in different cell cycle phases was calculated using ModFit LT for Mac (BD Biosciences).

Real-time Quantitative PCR—Total RNA extraction and real-time PCR analysis were performed as described previously (20). Briefly, total RNA was extracted from cells expressing FoxM1b WT or a FoxM1b mutant using TRIzol reagent (Invitrogen) according to the instructions of the manufacturer. Two-step real-time PCR was performed using cDNA prepared from RNA using a Superscript III first-strand cDNA synthesis kit (Invitrogen) and a SYBR Green PCR Master Mix (Applied Biosystems) on an ABI 7900 instrument following the instructions of the manufacturer. The thermal cycling conditions were 50 °C for 2 min, 95 °C for 10 min, 40 cycles of 95 °C for 15 s, and then 60 °C for 1 min. The -fold change in expression levels (using GAPDH as an internal control) was determined by a comparative Ct method using the formula 2−ΔΔCt, where Ct is the threshold cycle of amplification. The sequences of PCR primers were as follows: cyclin B1, tgggtgctgcctgttt (forward) and ccgaccgaccaaggtta (reverse); Aurora B, accctttgagagtgcatcac (forward) and gacgagtttggagatggct (reverse); PLK1, tgcggcgcctgctacta (forward) and ggcggttagctgggaagta (reverse); and GAPDH, acctgactggcttctaga (forward) and tccaccacccgcttgta (reverse).

Statistical Analysis—All experiments were performed at least three times in triplicates for each group. The results are presented as the mean ± S.D. Statistical significance was determined using Student’s t test, and the level of significance was set at p < 0.05.

RESULTS

FoxM1b Is Subject to SUMOylation—Because SUMOylation has emerged as an important mechanism for transcriptional control, we first examined whether FoxM1b is subject to SUMO modification. Interestingly, we found a series of bands migrating slower than FoxM1b. These bands were recognized by anti-Myc antibody in cells expressing Myc-tagged FoxM1b and SUMO-1 or SUMO-2/3, suggestive of SUMO modification, as well as multiple SUMO acceptor sites (Fig. 1A). Importantly, these higher molecular weight FoxM1b species reacted with anti-SUMO-1 and anti-SUMO-2/3 antibodies, confirming their identity (Fig. 1A). Notably, FoxM1b was preferentially SUMOylated by SUMO-1 compared with SUMO-2/3 (Fig. 1A). An in vitro SUMOylation assay verified that FoxM1b can be multi-SUMOylated by WT SUMO-1 but not a SUMO-1 mutant that has deletion of the two C-terminal glycine residues required for its conjugation to target proteins (Fig. 1B). Importantly, we also immunoprecipitated SUMOylated endogenous FoxM1b in cells under denaturing conditions, further confirming the covalent conjugation of FoxM1b with SUMO-1 (Fig. 1C). Taken together, these results indicate that FoxM1b is subject to SUMO modification.

We next used the SUMOplot™ analysis program to search for potential SUMO acceptor sites and identified six SUMOylation consensus motifs, kX(XE) (denotes a hydrophobic amino acid and X denotes any amino acid) (21), on FoxM1b. They are located at positions 201, 218, 341, 445, 463, and 480.
None of these are within the DNA-binding domain or transactivation domain (Fig. 1D). We subsequently used site-directed mutagenesis to mutate these six lysine residues to arginine, individually or in combination, to create FoxM1b-KR mutants that fail to be SUMOylated at specific sites. Only mutation of all six Lys residues to Arg (6KR) abolished SUMOylation (Fig. 1E and data not shown), indicating that all six sites are SUMO-1 acceptor sites.

**PLK1-mediated Phosphorylation of FoxM1b Antagonizes Its SUMOylation**—The phosphorylation of some proteins affects their SUMOylation, usually negatively (22–24). To determine the interplay between phosphorylation (mediated by PLK1) and SUMOylation of FoxM1b, we transfected U2OS cells with either FLAG-tagged WT, TD, or KD PLK1 and examined their effects on SUMO conjugation to FoxM1b. WT or TD PLK1 significantly decreased SUMOylation of FoxM1b, but KD PLK1 increased its SUMOylation (Fig. 2A), suggesting that PLK1 activity, directly or indirectly, modulates FoxM1b SUMOylation. To investigate whether PLK1-mediated phosphorylation of FoxM1b interferes with SUMOylation of FoxM1b, we transfected U2OS cells with SUMO-1 along with either Myc-tagged WT, a phosphomimetic mutant (EE), or an unphosphorylatable mutant (AA) of FoxM1b. The FoxM1b-EE mutant showed dramatically decreased SUMO modification compared with WT FoxM1b, whereas the FoxM1b-AA mutant showed the opposite (Fig. 2B). This suggests that PLK1-dependent phosphorylation of FoxM1b interferes with the SUMOylation of FoxM1b. To further demonstrate that PLK1 is directly involved in this process, bacterially expressed FoxM1b was subjected to an in vitro kinase assay using PLK1 as a kinase source, followed by an in vitro SUMOylation assay. PLK1-directed phosphorylation was confirmed by Western blot analysis with an anti-phospho-specific Ser(P)-724-FoxM1b antibody (Ser-724 on FoxM1b is a major PLK1 phosphorylation site (18)) (Fig. 2C). Upon phosphorylation of FoxM1b by PLK1 in vitro, SUMO conjugation to FoxM1b was reduced significantly compared with SUMOylation of unphosphorylated FoxM1b (Fig. 2D), which shows that PLK1 directly interferes with the SUMOylation of FoxM1b.

**SUMOylation Inhibits the Transcriptional Activity of FoxM1b, Which Is Antagonized by PLK1-mediated Phosphorylation**—To study the functional significance of SUMO modification of FoxM1b, we used a FoxM1b reporter construct in Dual-Luciferase assays to examine whether SUMOylation reg-
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FIGURE 2. PLK1-mediated phosphorylation of FoxM1b modulates FoxM1b SUMOylation. A, PLK1 activity modulates FoxM1b SUMOylation. U2OS cells were transfected with Myc-tagged FoxM1b and FLAG-tagged WT, TD, or KD PLK1. The SUMOylation of FoxM1b was examined by Western blotting using anti-Myc and anti-SUMO-1 antibodies. FLAG-tagged PLK1 expression was monitored using anti-FLAG antibody. B, PLK1-dependent phosphorylation of FoxM1b was inhibited by SUMO-1 in a dose-dependent manner (Fig. 3D). Although the FoxM1b-6KR mutant showed minimal transactivation, the FoxM1b-AA/6KR mutant had robust transactivation activity, which was close to that of the FoxM1b-EE mutant (Fig. 3D). These results, along with our observation that the FoxM1b-AA mutant showed increased SUMOylation (Fig. 1B), suggest that PLK1-induced activation of FoxM1b is indeed mediated through restricting its SUMO conjugation. C, PLK1-directed phosphorylation was determined by Western blot analysis with an anti-phospho-specific Ser(P)-724-FoxM1b antibody. D, following an in vitro kinase assay, those samples were analyzed using an in vitro SUMOylation assay. NS, non-specific band.

ulates transactivation by FoxM1b (18). The transactivation activity of FoxM1b was inhibited by SUMO-1 in a dose-dependent manner (Fig. 3A). In contrast, SUMO-2 did not exhibit a statistically significant effect on the transactivation activity of FoxM1b (data not shown). Furthermore, Ubc9 (the sole E2 conjugate enzyme) and SUMO-1 were transfected individually or together to further determine the effect of SUMO modification on the transactivation activity of FoxM1b. Although Ubc9 or SUMO-1 individually inhibited the transcriptional activity of FoxM1b, the combination of these two proteins further decreased the transactivation activity of FoxM1b (Fig. 3B). To further confirm that SUMOylation of FoxM1b leads to inhibition of its transactivation activity, SUMO-1 was fused to the N terminus of FoxM1b (a construct named SUMO-1-WT-FoxM1b). The covalent attachment of SUMO by linear gene fusion has been found to mimic the activity of SUMO conjugation (25–27). We then compared the transactivation activity of FoxM1b mutants (SUMOylation-deficient (6KR), SUMO-1-WT) with WT FoxM1b. Consistently, compared with WT FoxM1b, the FoxM1b-6KR mutant significantly increased transactivation activity, whereas SUMO-1-WT-FoxM1b exhibited dramatically reduced activity (Fig. 3C). The difference in their transactivation activity was not due to the changes of their protein levels because the protein levels of these mutants were similar to that of WT FoxM1b (Fig. 3C). These findings suggest that SUMOylation negatively modulates the transactivation activity of FoxM1b.

To determine whether PLK1-mediated activation of FoxM1b transactivation activity is mediated by antagonizing its SUMO modification, we compared the transactivation activity of the PLK1 phosphorylation-deficient and SUMOylation-deficient (AA/6KR) FoxM1b mutant with FoxM1b-AA and FoxM1b-EE mutants (Fig. 3D). Although the FoxM1b-AA mutant showed minimal transcriptional activity, the FoxM1b-AA/6KR mutant had robust transcriptional activity, which was close to that of the FoxM1b-EE mutant (Fig. 3D). These results, along with our observation that the FoxM1b-AA mutant showed increased SUMOylation (Fig. 1B), suggest that PLK1-induced activation of FoxM1b is indeed mediated through restricting its SUMO conjugation.

It has been reported previously that the transactivation activity of FoxM1b fluctuates in a cell cycle-dependent manner (8). We investigated the transcriptional activity of the FoxM1b-EE, FoxM1b-AA, FoxM1b-6KR, SUMO-1-WT, and SUMO-1-AA/6KR mutants throughout the cell cycle. The transcriptional activity of the FoxM1b-EE, FoxM1b-6KR, and FoxM1b-AA/6KR mutants was constitutively high in all phases of the cell cycle (Fig. 3E). In contrast, the transactivation by the FoxM1b-AA mutant and SUMO-1-WT-FoxM1b remained minimal throughout the cell cycle (Fig. 3E). These results suggest that the postranslational modifications mediated by SUMOylation and PLK1-induced phosphorylation contribute to the cell cycle-dependent regulation of FoxM1b.

PLK1-mediated Phosphorylation Promotes FoxM1b Nuclear Translocation via Interfering with Its SUMO Conjugation—We sought to elucidate the underlying mechanism by which the transcriptional activity of FoxM1b is activated by PLK1 but inhibited by SUMOylation. Nuclear translocation of FoxM1b is a general paradigm in its regulation. For a number of proteins, nuclear import/export depends on their SUMOylation (28–32). We tested whether cytoplasmic retention plays a role in FoxM1b transcriptional repression upon SUMOylation. Immunofluorescence staining was performed to visualize the subcellular localization of FoxM1b WT and mutants. The majority of the FoxM1b-6KR mutant was present in the nucleus, whereas less than half of WT FoxM1b resided in
the nucleus (Fig. 4A). In contrast, only about 13% of SUMO-1-WT-FoxM1b remained in the nucleus (Fig. 4A). FoxM1b-EE and FoxM1b-AA mutants exhibited subcellular localization that was similar to the FoxM1b-6KR mutant and SUMO-1-WT-FoxM1b, respectively (Fig. 4A). Furthermore, when the SUMO acceptor sites on the FoxM1b-AA mutant were blocked by mutating its six lysines to arginines (FoxM1b-AA/6KR), this resulted in its translocation from the cytoplasm to the nucleus (Fig. 4A). Subcellular fractionation assays further confirmed these results (Fig. 4B). Therefore, these findings suggest that SUMOylation of FoxM1b keeps FoxM1b in the cytoplasm, thereby inhibiting its nuclear transcriptional activity. PLK1-mediated phosphorylation antagonizes the SUMO conjugation to FoxM1b and promotes nuclear translocation, therefore escalating its transactivation activity.

SUMOylation of FoxM1b Affects Its Protein Stability—Protein SUMOylation has been known to regulate protein stability in both negative and positive fashions (33). To test the effect of SUMO modification on FoxM1b protein stability, we examined the decay rate of WT and mutant FoxM1b. WT or mutant FoxM1b-expressing U2OS cells were treated with cycloheximide, a protein translation inhibitor, for different times (0, 2, 4, 6, or 8 h), and the protein levels of FoxM1b were evaluated by Western blot analysis (Fig. 5A). Results from three independent experiments were quantified (Fig. 5A). In comparison to WT FoxM1b, the stability of SUMO-1-WT-FoxM1b and FoxM1b-AA were decreased, whereas the stability of FoxM1b-6KR and FoxM1b-EE mutants were increased substantially (Fig. 5A). Interestingly, the FoxM1b-AA/6KR mutant that is deficient in both SUMOylation and PLK1 phosphorylation showed...
increased stability, similar to that of the FoxM1b-6KR mutant (Fig. 5A). This result suggests that FoxM1b SUMOylation negatively regulates its protein stability and that this effect is inhibited by PLK1-mediated phosphorylation.

To determine whether FoxM1b SUMOylation facilitates its ubiquitination and subsequent degradation, U2OS cells were cotransfected with WT or a mutant (6KR, SUMO-1-WT, AA, EE, or AA/6KR) FoxM1b was examined by immunofluorescence staining using anti-Myc antibody. Quantification of a representative experiment is shown in the right panels. Similar results were obtained from three independent experiments. N, nucleus; C, cytoplasm. Scale bar = 10 μm. B, an additional set of samples was subjected to subcellular fractionation. The levels of exogenous FoxM1b in nuclear and cytoplasmic fractions were determined by immunoblotting with anti-Myc antibody. The relative purity of the nuclear and cytoplasmic fractions was confirmed by sequential probing for the nuclear marker Lamin A/C and the cytoplasmic marker α-tubulin.

FIGURE 4. PLK1-mediated phosphorylation promotes FoxM1b nuclear translocation by interfering with its SUMO conjugation. A, U2OS cells were transfected with Myc-tagged FoxM1b WT or a mutant (6KR, SUMO-1-WT, AA, EE, or AA/6KR). The cellular localization of ectopically expressed WT or mutant FoxM1b was examined by immunofluorescence staining using anti-Myc antibody. Quantification of a representative experiment is shown in the right panels. Similar results were obtained from three independent experiments. N, nucleus; C, cytoplasm. Scale bar = 10 μm. B, an additional set of samples was subjected to subcellular fractionation. The levels of exogenous FoxM1b in nuclear and cytoplasmic fractions were determined by immunoblotting with anti-Myc antibody. The relative purity of the nuclear and cytoplasmic fractions was confirmed by sequential probing for the nuclear marker Lamin A/C and the cytoplasmic marker α-tubulin.

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To determine whether FoxM1b SUMOylation facilitates its ubiquitination and subsequent degradation, U2OS cells were cotransfected with WT or mutant (6KR, SUMO-1-WT, AA, EE, or AA/6KR) Myc-tagged FoxM1b with or without His-ubiquitin. Cells were then treated with MG132 to block proteasomal degradation. WT FoxM1b showed a higher level of ubiquitination compared with the FoxM1b-6KR and FoxM1b-EE mutants (Fig. 5B). In contrast, we observed significantly enhanced ubiquitination of SUMO-1-WT-FoxM1b and FoxM1b-AA relative to their WT counterpart (Fig. 5B). The FoxM1b-AA/6KR mutant had ubiquitination levels similar to the FoxM1b-6KR or FoxM1b-EE mutant (Fig. 5B). These results suggest that FoxM1b SUMOylation facilitates the ubiquitination of FoxM1b and that, consequently, it substantially decreases the stability of FoxM1b, a common working mechanism by which SUMOylation negatively regulates the stability of its target protein.
It has been reported that FoxM1b is degraded during entry into anaphase (34). We hypothesized that FoxM1b is SUMOylated at the late stages of mitosis to facilitate its protein degradation. To test this hypothesis, we interrogated the occurrence of FoxM1b SUMOylation events during M phase of the cell cycle. HeLa cells stably expressing His6-tagged SUMO-1 were synchronized at prometaphase, metaphase, anaphase, and telophase. SUMOylation of the endogenous FoxM1b was then examined by immunoprecipitation and Western blot analysis (Fig. 6). SUMOylation of endogenous FoxM1b mainly occurred at anaphase and telophase, corresponding to the time at which FoxM1b undergoes proteasomal degradation.

PLK1-dependent Regulation of FoxM1b SUMOylation Is Required for Timely Entry into and Progress through M Phase—To explore the functional consequence of FoxM1b SUMOylation, a rescue assay was performed using U2OS cells stably expressing various WT and mutant forms of FoxM1b. Endogenous FoxM1b levels were depleted using shRNA that targeted the 3'-UTR of FoxM1b. Rescue of the FoxM1b knockdown phenotype was analyzed in cells stably expressing Myc-tagged FoxM1b-WT, FoxM1b-6KR, FoxM1b-SUMO-1-WT, FoxM1b-AA, FoxM1b-EE, and FoxM1b-AA/6KR. FACS analysis showed a dramatic increase in 4N cells upon FoxM1b depletion (Fig. 7A), indicative of defects in G2/M transition and/or mitotic progression. To determine whether an increase in 4N cells is due to a delay in G2/M transition and/or an increase in binucleated cells (indicative of a defect in cytokinesis), we scored binucleated cells (Fig. 7B). A delay of cells in G2/M transition and an increase in the number of binucleated cells (9.3%) seems to account for the accumulation of cells with 4N DNA content in the absence of FoxM1b (Fig. 7, A and B). Similar to WT FoxM1b, expression of FoxM1b-6KR and FoxM1b-EE restored normal cell cycle progression (Fig. 7A). However, the SUMO-1-WT–FoxM1b and FoxM1b-AA mutants were not able to rescue these phenotypes. Interestingly, cells expressing the FoxM1b-AA/6KR mutant acted similarly as those harboring the FoxM1–6KR mutant (Fig. 7, A and B). Taken together, these results suggest that SUMOylation of FoxM1b leads to mitotic defects and that PLK1-dependent regulation of FoxM1b SUMOylation ensures timely entry into and progress through M phase.

To provide an in vivo correlation, we also examined the expression of FoxM1b target genes in the context of the rescue assay described above. Although the mRNA and protein levels of FoxM1 target genes, including Aurora B, PLK1, and cyclin B1, were down-regulated significantly in FoxM1-depleted cells, expression of WT FoxM1b rescued this down-regulation, as measured by quantitative RT-PCR and Western blot analysis (Fig. 7, C and D). Furthermore, we observed elevated expression of FoxM1 target genes in cells expressing the FoxM1–6KR, FoxM1–EE, or FoxM1–AA/6KR mutant (Fig. 7, C and D). In contrast, Aurora B, PLK1, and
cyclin B1 levels remained low in cells expressing the SUMO-1-WT-FoxM1b or FoxM1b-AA mutant (Fig. 7, C and D). The expression of the FoxM1b target genes positively correlated with their transcriptional activities and protein stability of FoxM1b in those cells (Figs. 4 and 5).

DISCUSSION

Accumulating evidence demonstrates that FoxM1b is an important transcription factor that plays a vital role in many cellular processes, including cell differentiation, proliferation, cellular transformation, angiogenesis, apoptosis, and metastasis. Therefore, this key protein is under tight regulation. Post-translational modifications of the protein have shown to contribute significantly to the regulation of FoxM1b activity and function. For instance, we showed previously that PLK1 directly interacts with and phosphorylates FoxM1b, leading to full activation of its transcription activity at G2/M transition (18). This activation event, in turn, controls the expression of a large array of G2/M regulators that are essential for timely mitotic entry and progression (18). We now demonstrate the underlying molecular mechanism by which PLK1-mediated phosphorylation results in the increased transactivation capability of FoxM1b. We demonstrate that FoxM1b is subject to SUMO modification, which leads to its cytoplasmic retention and ubiquitination and subsequent degradation in the cytoplasm. Such a posttranslational modification leads to loss of FoxM1b transcriptional activity and severe defects in mitosis. PLK1-mediated phosphorylation can interfere with SUMO conjugation to FoxM1b, thereby keeping FoxM1b active and ensuring the timely and smooth mitotic entry and progression.

We have provided in vitro and in vivo evidence that FoxM1b can be SUMOylated, preferentially by SUMO-1. Most transcription factors that are SUMOylated show reduced transactivation capacity when conjugated to SUMO-1. Indeed, our results show that, when FoxM1b is modified by SUMO-1, its transcriptional activity is decreased significantly. Conversely, blocking SUMO acceptor sites leads to a dramatic increase in the transactivation capability of FoxM1b.

Interestingly, we found that the SUMO modification event is negatively regulated by PLK1. PLK1-mediated phosphorylation inhibits SUMO conjugation to FoxM1b, thereby relieving the inhibitory effect induced by SUMOylation, as occurs with c-Jun (24), p53 (24), IκBα (22), and Mdm2 (35). We consistently observed that the kinetics of SUMOylation of FoxM1b are contrary to the expression and activity patterns of PLK1. The SUMOylation of FoxM1b occurs mainly late in mitosis (anaphase and telophase), whereas the level and activity of PLK1 are maximal in early mitotic phases (prophase and metaphase) and decay quickly late in mitosis. Our data showing that SUMOylation of FoxM1b by SUMO-1 represses FoxM1b transcriptional activity contradict a recent report that demonstrated SUMO-dependent activation of FoxM1b (36). A similar controversy has been reported regarding the functional impact of SUMO modification on another FoxM1 isoform, FoxM1c (37, 38). One group reported activation of FoxM1c by SUMOylation, whereas the other reported inhibition. These contradictions may reflect the complexity of FoxM1 regulation, where SUMOylation of FoxM1 could play either an inhibitory or activating role under different physiological conditions and experimental systems.
Nuclear-cytoplasmic shuttling is a general paradigm in the regulation of transcription factors. We demonstrate that SUMOylation of FoxM1b affects its cellular trafficking. We have provided evidence that SUMO modification promotes its cytoplasmic retention, which would suppress the nuclear transcriptional activity of FoxM1b. Our findings that the FoxM1b-EE and FoxM1b-AA mutants exhibited subcellular localization similar to the FoxM1b-6KR mutant and SUMO-1-WT-FoxM1b, respectively, and that blocking the SUMO acceptor sites on the FoxM1b-AA mutant resulted in its translocation from the cytoplasm to the nucleus would support the notion that PLK1-mediated phosphorylation antagonizes SUMOylation of FoxM1b.

**FIGURE 7.** PLK1-dependent regulation of FoxM1b SUMOylation is required for timely entry into and progress through M phase. U2OS cells stably expressing empty vector (EV), Myc-tagged FoxM1 WT, or a mutant (6KR, SUMO-1-WT, AA, EE, or AA/6KR) were infected with lentivirus encoding FoxM1b shRNA (targeting the 3′-UTR) to knock down endogenous FoxM1b. A, cell cycle distributions were determined by flow cytometry analysis 72 h after infection. PI, propidium iodide. B, the percentage of binucleated cells was determined on the basis of DAPI and α-tubulin staining 72 h post-infection. C, cell lysates were collected 72 h after infection and analyzed by immunoblotting. Blotting for α-tubulin was used as a loading control (Ctrl). D, total RNA was extracted 48 h after transfection and subjected to RT-PCR analysis. RT-PCR for GAPDH was used as an internal control.
PLK1 Antagonizes SUMO Conjugation to FoxM1b

SUMO modification of FoxM1b and promotes nuclear translocation, resulting in enhanced transactivation activity. Interestingly, one of the SUMO conjugation sites, Lys-341, is located within a nuclear localization sequence (residues 335–353, RRNMTIKTELPLGARRKMK) on FoxM1b (39). It is likely that SUMOylation may disturb the recruitment of components of the nuclear transport machinery to the nuclear localization sequence on FoxM1b. Nevertheless, further investigation is needed to elucidate the exact working mechanism by which SUMOylation affects the cellular trafficking of FoxM1b.

It has been long believed that SUMOylation is not involved in proteolytic targeting. Instead, SUMO can act, in some cases, as an antagonist of ubiquitin during proteasome-mediated degradation (33). However, recent studies demonstrated that SUMOylation can function as a secondary signal mediating ubiquitin-dependent degradation by the proteasome (40). For instance, the promyelocytic leukemia protein (PML) is ubiquitinated by RNF4 in a SUMO-dependent manner (41). SUMOylation of PML promotes its recognition by the SUMO interaction motifs on RNF4, which triggers ubiquitination and degradation of PML. In this study, we found that SUMOylation of FoxM1b results in significantly reduced FoxM1b stability by facilitating ubiquitin-mediated proteasomal degradation. Studies have shown that FoxM1 is actively degraded during exit from mitosis by the anaphase-promoting complex (or cyclosome) (APC/C) and its cofactor Cdh1 (34). GPS-SBM 1.0 software predicts two putative SUMO interaction motifs on Cdh1 that are located at residues 362–365 (VITV) and residues 391–394 (VVIT). It is tempting to speculate that FoxM1b may be ubiquitinated by APC/C-Cdh1 in a SUMO-dependent fashion, as in the case of the aforementioned PML. The interplay between the SUMOylation and ubiquitination of FoxM1b awaits determination.

In agreement with the finding that SUMO modification facilitates the ubiquitin-mediated degradation of FoxM1b, we also found that SUMOylation of FoxM1b is very low in early mitosis and high in late mitosis, which is contrary to the level and activity of PLK1. Therefore, we came up with the following model describing the interplay between phosphorylation (mediated by PLK1) and SUMOylation of FoxM1b (Fig. 8). During G2/M phase, PLK1 phosphorylates FoxM1b, which prevents it from being SUMOylated and leads to robust activation of FoxM1b transcriptional activity, which is required for entry into and progression through mitosis. During the exit from mitosis, the level and activity of PLK1 drop rapidly, which relieves the inhibitory effect on SUMO conjugation to FoxM1b. Subsequently, SUMOylated FoxM1b relocates to the cytoplasm, where it is ubiquitinated and degraded by the proteasome. This, in turn, leads to the turning off of the transcription of mitotic regulators that are controlled by FoxM1b and allows the cell to exit mitosis.

In summary, our data show that the posttranslational modification of FoxM1b by SUMO-1 conjugation can lessen its transactivation capacity and protein stability. PLK1 prevents such a modification, which leads to full activation of FoxM1b and timely G2/M transition and mitotic progression. Uncovering the underlying mechanism by which PLK1 activates the activity of FoxM1b would greatly facilitate therapeutic interventions that focus on targeting the PLK1-mediated and/or FoxM1-mediated signaling network.

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