Published in final edited form as:
Nat Cell Biol. 2008 September ; 10(9): 1076–1082.

Plk1-dependent phosphorylation of FoxM1 regulates a transcriptional programme required for mitotic progression

Zheng Fu[1,3], **Liviu Malureanu**[2,3], **Jun Huang**[4], **Wei Wang**[5], **Hao Li**[5], **Jan M. van Deursen**[2,3], **Donald J. Tindall**[1,6], and **Junjie Chen**[4,6]

[1]Department of Urology, Mayo Clinic College of Medicine, Rochester, Minnesota 55905, USA.

[2]Department of Pediatrics, Mayo Clinic College of Medicine, Rochester, Minnesota 55905, USA.

[3]Department of Biochemistry and Molecular Biology, Mayo Clinic College of Medicine, Rochester, Minnesota 55905, USA.

[4]Department of Therapeutic Radiology, Yale University School of Medicine, New Haven, Connecticut 06520-8040, USA.

[5]Department of Chemistry and Chemical Biology, University of New Mexico, Albuquerque, New Mexico 87131, USA.

Abstract

Proper control of entry into and progression through mitosis is essential for normal cell proliferation and the maintenance of genome stability1⁻4. The mammalian mitotic kinase Pololike kinase 1 (Plk1) is involved in multiple stages of mitosis5. Here we report that Forkhead Box M1 (FoxM1), a substrate of Plk1 (refs 6⁻8), controls a transcriptional programme that mediates Plk1-dependent regulation of cell-cycle progression. The carboxy-terminal domain of FoxM1 binds Plk1, and phosphorylation of two key residues in this domain by Cdk1 is essential for Plk1–FoxM1 interaction. Formation of the Plk1–FoxM1 complex allows for direct phosphorylation of FoxM1 by Plk1 at G2/M and the subsequent activation of FoxM1 activity, which is required for expression of key mitotic regulators, including Plk1 itself. Thus, Plk1-dependent regulation of FoxM1 activity provides a positive-feedback loop ensuring tight regulation of transcriptional networks essential for orderly mitotic progression.

Transitions through cell-cycle phases require the coordination of multiple events and are tightly regulated by protein kinases[1,2,5]. In addition, transcriptional control of key cell-cycle regulators are important for cellcycle progression[9,10]. Thus, defining the links between protein kinases and transcriptional networks is essential for an understanding of normal cell-cycle progression and how specific factors may contribute to the misregulation of this process in diseases.

In *Saccharomyces cerevisiae*, the polo kinase Cdc5p coordinates cellcycle-dependent transcription at G2/M by directly phosphorylating and regulating the activity of a co-activator

[6]Correspondence should be addressed to J.C. or D.J.T (e-mail: junjie.chen@yale.edu; tindall.donald@mayo.edu).

AUTHOR CONTRIBUTIONS Z.F. performed most of experiments, analysed the data and wrote the paper; L.M. and J.M.V. analysed the time-lapse imaging data; J.H. performed the experiments shown in Fig. S2a and b; W. W. and H. L. synthesized ON01910; Z.F. and J.C. designed the experiments; J.C. and D.J. T. supervised the study and revised the paper.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

of the forkhead transcription family member Fkh2p (ref. 11). The mammalian Polo-like kinase 1 (Plk1) is also essential for multiple events throughout M phase[1,5] and several Plk1 targets involved in mitosis have been identified[12–17]. So far, no direct link between Plk1 and the transcription factors controlling gene expression has been identified, although the conservation of functions between polo kinases in yeast and higher eukaryotes makes it tempting to speculate that Plk1 may have a role in mammalian cells similar to that recently demonstrated for Cdc5p in yeast[11].

The mammalian transcription factor Forkhead Box M1 (FoxM1) is important in regulating mitotic entry and subsequent execution of the mitotic programme by controlling the expression of a cluster of G2/M target genes[6,8,18]. However, the mechanisms regulating the activation of FoxM1 at specific cell-cycle phases, and how this contributes to G2/M progression are largely unknown.

Here, using yeast two-hybrid screening, we identified FoxM1 as a direct binding partner of Plk1. Six of the twenty-four positive clones encoded various lengths of the FoxM1 C terminus, with the smallest clone encoding residues 463–748 of FoxM1. These initial results suggest that there is a link between Plk1 and a transcription factor controlling a G2/M transcriptional programme.

In vitro translated FoxM1 associated with GST-fused Plk1 (GST–Plk1), but not with GST (Fig. 1a), indicating that Plk1 binds directly to FoxM1. In contrast, bacterially expressed FoxM1 failed to interact with *in vitro* translated Plk1 (Supplementary Information, Fig. S1a), suggesting that post-translational modification of FoxM1 may be required for this interaction. In addition, ectopically expressed FoxM1 formed a complex with endogenous Plk1 (Fig. 1b), but not Plk2 or Plk3 (Supplementary Information, Fig. S1c). Plk1 and FoxM1 show periodic expression patterns, with both proteins increasing markedly in G2/M cells[19] (Supplementary Information, Fig. S1b). Indeed, an endogenous Plk1–FoxM1 complex was readily observed in G2/M cells, whereas this complex was barely detectable in asynchronous cells (Fig. 1c).

A noticeable progressive decrease in FoxM1 mobility was observed when cells were released from G0/G1 arrest (Supplementary Information, Fig. S1b), indicating that FoxM1 undergoes an initial phosphorylation in S phase followed by hyperphosphorylation during G2/M, a time at which Plk1 activity is maximal. Spatial and temporal coordination of Plk1 activity is mediated by the C-terminal Polo-box domain (PBD) of Plk1 (refs 20–22). Furthermore, the PBD is required for recruitment of Plk1 to its substrates that have been 'primed' by phosphorylation at specific docking sites23. Therefore, FoxM1 may undergo an initial priming phosphorylation, allowing it to be recruited and directly phosphorylated by Plk1.

Plk1 PBD recognizes a consensus sequence of S-pS/pT-P/X22,[24]. FoxM1 sequence contains two potential PBD-binding sites (Thr 596 and Ser 678). Our yeast two-hybrid results indicated that a C-terminal domain of FoxM1 (residues 463–748) is essential for Plk1 binding. Therefore, we examined whether Thr 596 and Ser 678 of FoxM1 would mediate the interaction between these two proteins. Thr 596 and Ser 678 were mutated to Ala individually ($FoxM1^{T596A}$, $FoxM1^{S678A}$) or in combination ($FoxM1^{TSAA}$), and their ability to bind GST-tagged Plk1 PBD was analysed. $FoxM1^{WT}$ associated with GST–PBD, but not with GST or the GST–PBD mutant (Fig. 1d). Binding to the Plk1 PBD in the $FoxM1^{T596A}$ and $FoxM1^{S678A}$ single mutants was reduced; however, mutation of both residues abolished this interaction (Fig. 1d). Thus, both Thr 596 and Ser 678 of FoxM1 seem to be key sites involved in a phosphorylation-dependent interaction between FoxM1 and Plk1 PBD.

Many docking sites on Plk1-binding proteins are initially phosphorylated by Cdk1/cyclin B. FoxM1 preferentially binds to the Cdk1/cyclin B complex in G2, and Thr 596 of FoxM1 was previously identified as a Cdk1 phosphorylation site[7]. We determined whether phosphorylation

of FoxM1 by Cdk1 is responsible for the formation of Plk1–FoxM1 complex. We performed pulldown assays using bacterially expressed GST–Plk1 PBD and MBP–FoxM1 fusion proteins, with or without Cdk1/ cyclin B. Phosphorylation of MBP–FoxM1 by Cdk1 promoted a strong interaction between FoxM1 and Plk1 PBD (Supplementary Information, Fig. S2a). Inhibition of Cdk1, but not Plk1 (ref. 25), markedly reduced the binding of endogenous FoxM1 to GST–Plk1 PBD (Supplementary Information, Fig. S2b, e). Thus, Cdk1-dependent phosphorylation of FoxM1 is required for its interaction with Plk1.

FoxM1 is hyperphosphorylated during G2/M phase, which correlates with its transcriptional activity being cell-cycle-restricted. Therefore, we hypothesized that Plk1 phosphorylates and activates FoxM1 during G2/M transition. We found that FoxM1 was phosphorylated by the constitutively active Plk1 ($Plk1^{TD}$) *in vitro*, but not by the catalytically inactive $Plk1^{KD}$ mutant (Fig. 2a).

We next sought to map the major Plk1 phosphorylation sites on FoxM1. A series of FoxM1deletion-mutants were generated and tested for their capacity to be phosphorylated by Plk1 *in vitro*. Although the amino-terminal segments of FoxM1 were weakly phosphorylated by Plk1, C-terminal segments, especially those containing the transcriptional activation domain (TAD, residues 617–748; Supplementary Information, Fig. S3a, b), showed much higher levels of phosphorylation.

Plk1 substrates do not in general contain a strict consensus phosphorylation motif[26,27]. Therefore, we used mass spectrometry analysis for the identification of Plk1-dependpent FoxM1 phosphorylation sites. Using recombinant FoxM1 protein purified from bacteria and phosphorylated *in vitro* by Plk1, we identified four Ser residues (Ser 680, Ser 702, Ser 715 and Ser 724) located within the TAD of FoxM1 as potential Plk1 phosphorylation sites. Single- and double-site mutants were generated and their capacity to be phosphorylated by Plk1 was determined *in vitro*. Single mutation of Ser 715 or Ser 724 reduced Plk1-dependent phosphorylation of FoxM1 (Supplementary Information, Fig. S3c), whereas phosphorylation was almost abolished when the S715A;S724A double mutant was used (Fig. 2b). Thus, Ser 715 and Ser 724 seem to be the two major Plk1-dependent phosphorylation sites within the TAD region of FoxM1. Importantly, these two Ser residues, as well as the surrounding amino acids, are highly conserved among vertebrates (Fig. 2c), indicating that the phosphorylation of these two sites may have an evolutionarily conserved role in regulating FoxM1 activity.

We next determined whether FoxM1 is phosphorylated by Plk1 *in vivo*. We generated phospho-specific anti-FoxM1 antibodies. As shown in Fig. 2d, Both the anti-p715 and anti-p724 antibodies immunoreacted with wild-type FoxM1; however, only the anti-p715 antibody recognized the $FoxM1^{S724A}$ mutant and *vice versa*. Furthermore, neither antibody detected λ-phosphotase-treated wild-type FoxM1 (Supplementary Information, Fig. S3d). Thus, the anti-p715 and anti-p724 antibodies are highly specific and only recognize their corresponding targets. Using these phospho-specific antibodies, we showed that phosphorylation of endogenous FoxM1 could be readily detected in G2/M but not in asynchronous HeLa cells (Fig. 2e), indicating that FoxM1 is phosphorylated at Ser 715 and Ser 724 sites at G2/M. To determine whether Plk1 is required for these phosphorylation events, we depleted endogenous Plk1 by siRNA and observed a marked reduction of FoxM1 phosphorylation at Ser 715 and Ser 724 sites (Fig. 2f), supporting the suggestion that Plk1 is the kinase that phosphorylates FoxM1 *in vivo*.

We directly examined the possibility that Cdk1 could function as a priming kinase of FoxM1. To test this, we performed *in vitro* kinase assays using Cdk1, Plk1, or both as kinase sources. Consistent with earlier studies[7], Cdk1 could phosphorylate FoxM1 to some extent (Supplementary Information, Fig. S2c). Interestingly, in the presence of Cdk1 the

phosphorylation of FoxM1 by Plk1 was markedly elevated when compared with Plk1 alone. Furthermore, Cdk1-mediated phosphorylation was initiated as early as S/G2 phase *in vivo*, whereas Plk1-mediated phosphorylation occurred later, mainly at G2/M phases (Supplementary Information, Fig. S2d), suggesting that FoxM1 undergoes an initial priming phosphorylation by Cdk1, which allows its subsequent phosphorylation by Plk1.

To better understand the functional significance of Plk1-dependent phosphorylation of FoxM1, we tested whether Plk1 regulates FoxM1 transcriptional activity, assayed using the 6× *FoxM1*–TATA–luciferase reporter plasmid[7]. Wild-type Plk1, but not the kinase-inactive mutant of Plk1, significantly upregulated FoxM1 transcriptional activity in a concentration-dependent manner (Fig. 3a), suggesting a direct regulation of FoxM1 by Plk1-dependent phosphorylation.

We next investigated whether the association between Plk1 and FoxM1 is necessary for Plk1-dependent activation of FoxM1. Mutation of one of the two residues involved in the docking of Plk1 to FoxM1 resulted in only a partial decrease in Plk1-enhanced FoxM1 transcriptional activity (T596A, 55%; S678A, 30%). In contrast, mutation of both residueled to a marked reduction (>90% decrease) in Plk1-dependent FoxM1 transcriptional activity (Fig. 3b). These results are consistent with those shown in Fig. 1d and indicate that phosphorylation of either Thr 596 or Ser 678 of FoxM1 can independently mediate the binding to Plk1 and this binding is essential for Plk1-dependent activation of FoxM1.

To test the hypothesis that Plk1-mediated phosphorylation of FoxM1 enhances its transcriptional activity, we mutated the two major Plk1 phosphorylation sites of FoxM1 (Ser 715 and Ser 724) to Ala ($FoxM1^{AA}$) or to glutamic acid ($FoxM1^{EE}$) and examined the effects on FoxM1 transcriptional activity. We included a FoxM1 mutant lacking the TAD ($FoxM1^{1–616}$) as a negative control in these experiments. Indeed, the phospho-mutant of FoxM1 was not activated by Plk1 (Fig. 3c), whereas $FoxM1^{EE}$ showed robust transcriptional activity with or without Plk1 (Fig. 3c). Taken together, these results indicate that after an initial priming phosphorylation of FoxM1 by Cdk1, Plk1 is recruited to and directly phosphorylates FoxM1, thereby enhancing FoxM1 transcriptional activity.

To address the functional relevance of Plk1-dependent FoxM1 phosphorylation *in vivo*, we developed a rescue assay using U2OS cells stably expressing various wild-type or mutant forms of FoxM1. Endogenous FoxM1 levels were depleted using siRNA and rescue of the *FoxM1* knock down phenotype was analysed in cells stably expressing Myc-tagged wild-type FoxM1 or siRNA-resistant Myc-tagged wild-type (WT-r) or S715A;S724A mutant (AA-r) FoxM1. As controls, U2OS cells stably transfected with empty vector were treated with siRNAs targeting FoxM1 or the firefly luciferase gene. Endogenous FoxM1 and Myc-tagged wild-type FoxM1 were significantly depleted but WT-r and AA-r remained abundant (Fig. 4b, Supplementary Information, Fig. S4a), confirming that the siRNA-resistant FoxM1 constructs were effective.

Previous studies have shown that cells lacking FoxM1 have multiple cellcycle defects, including a delay in G2 and reduced expression of G2/M FoxM1 target genes. Although some FoxM1-depleted cells eventually enter mitosis, additional mitotic defects may occur, resulting in the formation of polyploid and aneuploid cells[6,8]. Indeed, FACS, morphological analyses and time-lapse microscopy showed an increase in 4N cells and an accumulation of polyploid (>4N) cells (Fig. 4a; Supplementary Information, Fig. S4a, c) after FoxM1 depletion. Numerous mitotic defects, including misaligned and/or mis-segregated chromosomes, aberrant furrow formation, failed cytokinesis and prolonged mitotic progression, were also observed, confirming that FoxM1 is required for G2/M transition and proper mitotic progression. A delay of cells in G2/M transition and an increase in the number of binucleated cells (10.4%, Supplementary Information, Fig. S4b) seems to account for the accumulation of cells with 4N

DNA content in the absence of FoxM1. Expression of wild-type and siRNA-resistant wild-type FoxM1 partially or completely restored normal cell-cycle progression (Fig. 4a). However, the siRNA-resistant FoxM1 S715A;S724A mutant was not able to rescue these phenotypes, suggesting that Plk1-dependent phosphorylation and activation of FoxM1 is crucial for FoxM1 function at G2/M.

To provide an *in vivo* correlation, we next examined the expression of FoxM1 target genes, in the context of the rescue assay described above. Indeed, in FoxM1-depleted cells, we observed downregulation of FoxM1 target genes, including *Plk1*, *Cyclin B1*, and *Aurora B*, all of which are required for mitotic progression; expression of siRNA-resistant wild-type FoxM1 rescued this downregulation (Fig. 4b, c). In contrast, *Plk1*, *Cyclin B1* and *Aurora B* levels remained low in cells expressing the siRNA-resistant FoxM1 S715A;S724A mutant cells (Fig. 4b, c). Next, we blocked Plk1 activity using a chemical inhibitor[25]. Although inhibition of Plk1 activity reduced the expression of downstream targets of FoxM1, no effect was observed in cells expressing the constitutively active FoxM1^{EE} mutant (Fig. 4d). Moreover, time-lapse imaging analysis revealed that inhibition of Plk1 activity leads to mitotic defects, such as prolonged early mitotic phases and prometaphase arrest in wild-type cells. These defects were partially rescued in cells expressing FoxM1^{EE} (Fig. 4d; Supplementary Information, Movies 1, 2). Together, these data indicate that Plk1 regulates FoxM1 transcriptional activity by direct phosphorylation and thereby controls execution of a transcriptional programme required for mitotic progression.

We have developed a model to describe the role of Plk1-mediated regulation of FoxM1 at G2/M transition (Fig. 4e). This suggests that at late S and G2 phase, FoxM1 is initially phosphorylated by Cdk1 kinases, creating docking sites for the PBD of Plk1. Subsequently, Plk1 binds and directly phosphorylates FoxM1. This activates FoxM1 transcriptional activity, resulting in the enhanced expression of key mitotic regulators. As *Plk1* is a target gene of FoxM1, this mode of regulation can generate a positive-feedback loop, leading to a further increase in Plk1 levels and FoxM1 activity. Furthermore, as the major transcription factor during G2/M transition, full activation of FoxM1 ensures the coordinated expression of transcriptional networks essential for timely entry into M phase. This mode of regulation is reminiscent of the Rb-E2F pathway during G1/S transition, where Rb is hyperphosphorylated and releases E2F, allowing for transactivation of target genes that promote S phase entry[28].

The polo kinase Cdc5p also regulates a G2/M transcriptional programme in *S.cerevisiae*[11]. Here we have extended these observations and revealed a link between Plk1 and regulation of a G2/M transcriptional network in mammalian cells. In contrast to yeast Cdc5p-mediated phosphorylation of a co-activator Ndd1p, Plk1 directly phosphorylates and thereby activates FoxM1 transcriptional activity. Our data do not exclude the possibility that other transcription factor(s) may collaborate with FoxM1 to coordinate the appropriate expression of transcriptional networks. As various cell-cycle kinases are upregulated in many cancers, the results presented here should help to provide further insights into the link between aberrant expression of these kinases and alterations in cell proliferation as well as genomic integrity, potentially through misregulation of transcriptional programmes.

METHODS

Plasmid constructs siRNA oligonucleotides, RT–PCR and antibodies

Details of plasmid constructs, siRNA and primer sequences used in this study can be found in Supplementary Information Methods. Rabbit polyclonal antibodies recognizing phosphorylated Ser 678, phosphorylated Ser 715 or phos-phorylated Ser 724 of FoxM1 were raised against peptides CPFGNSpSPSDID, DTMNDpSLSKILLDC and KILLDIpSFPGLDC, respectively, and affinity-purified using their corresponding peptide columns. Other antibodies

used in this study were polyclonal anti-FoxM1 (1:200) and anti-Cyclin B1 (1:1000) (Santa Cruz Biotechnology), monoclonal anti-Plk1 (1:1000; Zymed), monoclonal antiMyc (1:1000; Invitrogen), monoclonal anti-Aurora B (1:250; BD Biosciences), and anti-β-actin (1:5000; Sigma) antibodies.

Cell culture and synchronization

293T and HeLa cells were cultured in RPMI 1640 medium (Invitrogen) containing 10% fetal bovine serum (FBS; Biosource), 100 units ml^{-1} penicillin, 100 units ml^{-1} streptomycin and 0.25 μg ml^{-1} amphotericin B (Fungizone; Invitrogen). U2OS cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) containing 10% FBS, 100 units ml^{-1} penicillin, 100 units ml^{-1} streptomycin and 0.25 μg ml^{-1} amphotericin B. For cell synchronization, T24 cells were arrested in G0 by contact inhibition. After at least 3 days of confluence, the cells were split 1:10 by seeding in multiple 100-mm dishes at a concentration of 10^6 cells per dish. Cells were collected at various time points after replating[30]. For synchronization of U2OS cells, cells were treated with 2 mM thymidine for 24 h and then released in fresh medium for 10 h. To arrest exponentially growing HeLa or U2OS cells at prometaphase, nocodazole was added to a final concentration of 100 ng ml^{-1} for 16–24 h. Mitotic cells were collected by mechanical shake-off.

Western blotting and immunoprecipitation

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 co-immunoprecipitation experiments, cleared lysates (1–2 mg protein) were immunoprecipitated with the indicated polyclonal antibodies and protein A-Sepharose for 1 h. In phosphatase treatment experiments, FoxM1 immunoprecipitates were treated with 400 units of λ phosphatase in the accompanying buffer (New England Biolabs) for 1 h at 30 °C. Proteins were separated on 7.5% SDS–PAGE, transferred to Immobilon P membranes and immunoblotted with the indicated antibodies.

GST pull-down assays

Human FoxM1 was transcribed and translated *in vitro* using TNT quick-coupled transcription/translation system (Promega). GST–Plk1 or GST control bound to glutathione-sepharose resin was incubated with *in vitro* translated and labelled with FoxM1 protein for 1 h at 4 °C, and the washed complexes were boiled in SDS–PAGE sample. Alternatively, glutathione-sepharose resin-coupled GST–Plk1 PBD wild-type, mutant or the GST control were incubated with cell lysates containing Myc-tagged wild-type or mutant FoxM1 for 1 h at 4 °C The resins were washed four times using the NETN buffer. Resin-bound complexes were eluted by boiling, separated by SDS–PAGE, and analysed by autoradiography or western blotting.

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. Slides were incubated with primary antibodies for 20 min at 37 °C. Fluorescein isothiocyanate-conjugated goat anti-rabbit and/or rhodamine-conjugated goat anti-mouse serum (Jackson ImmunoResearch) were used as secondary antibodies. All antibodies were diluted in 5% goat serum. Cells were counterstained with 4–6-diamidino-2-phenylindole (DAPI) dye for 30 s.

Protein kinase production and kinase assays

Constitutively active human ($Plk1^{TD}$), wild-type or kinase-defective ($Plkl^{KD}$) Plk1 were expressed in the baculovirus/insect cell system. Cdk1/cyclin B proteins were purchased from Invitrogen. GST–FoxM1 and its deletion mutant proteins were expressed in *Escherichia coli*

BL21 strain. Kinase and substrates (FoxM1 proteins) were incubated in kinase buffer (20 mM Hepes, pH 7.4, 150 mM KCl, 10 nM $MgCl_2$, 1 mM EGTA, 0.5 mM dithiothreitol, 5 mM NaF, 10 μM ATP, 5 μCi γ-^{32}P) for 30 min at 30 °C. Reactions were stopped by the addition of SDS sample buffer. Then samples were heated for 5 min to 95 °C before analysis by SDS–PAGE and autoradiography.

Luciferase assays

U2OS cells were transfected with the indicated plasmids using Lipofectamine 2000 according to manufacturer's protocol. Luciferase activity was determined 24 h after transfection using the dual luciferase kit (Promega). Luciferase levels were normalized to *Renilla* luciferase activity. Relative luciferase activity was expressed as percentage induction of promoter activity by the FoxM1 expression vector and Plk1, where promoter activity resulting from transfection with FoxM1 wild-type was set at 100%. Experiments were performed in triplicate and statistical analysis was performed using Microsoft Excel tools.

FACScan analysis

Cells were collected, washed with PBS and fixed with icecold 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^{-1} and propidium iodide at 50 μg ml^{-1}, and analysed on a FACScan flow cytometer (Becton Dickinson). The percentage of cells in different cell-cycle phases was calculated using ModFit LT for Mac (BD Biosciences).

Time-lapse microscopy

Experiments were performed using a Zeiss Axio Observer system with: CO_2 Module S, TempModule S, Heating Unit XL S, Pln Apo ×10/1.4 DICIII objective, AxioCam MRm camera and AxioVision 4.6 software. The imaging medium was DMEM containing 10% FBS. The temperature of the imaging medium was kept at 37 °C. The exposure times was 400 ms at 2 × 2 binning. Total time of mitosis was defined as the interval between NEBD (nuclear envelope breakdown) and cytokinesis. Mitotic defects were scored.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We are grateful for extensive discussions with R.H. Medema and J. Laoukili and their input. We thank H.M. Thompson for editing the manuscript. Mass spectrometry analysis was performed by Taplin Biological Mass Spectrometry Facility at Harvard University. This work was supported in part by grants from the National Institutes of Health (NIH RO1 CA113381 to JC), Mayo SPORE P50 (CA116201 project no. 1 to J.C.) and the T.J. Martell Foundation (to D.J.T.). J.C. is a recipient of an Era of Hope Scholars award from DOD. Z.F. is a recipient of a Ruth L. Kirschstein NRSA individual Fellowship from NIH.

References

1. Nigg EA. Mitotic kinases as regulators of cell division and its checkpoints. Nature Rev. Mol. Cell Biol 2001;2:21–32. [PubMed: 11413462]
2. Norbury C, Nurse P. Animal cell cycles and their control. Annu. Rev. Biochem 1992;61:441–470. [PubMed: 1497317]
3. Pines J, Rieder CL. Re-staging mitosis: a contemporary view of mitotic progression. Nature Cell Biol 2001;3:E3–E6. [PubMed: 11146636]
4. Murray AW. Recycling the cell cycle: cyclins revisited. Cell 2004;116:221–234. [PubMed: 14744433]

5. Barr FA, Sillje HH, Nigg EA. Polo-like kinases and the orchestration of cell division. Nature Rev. Mol. Cell Biol 2004;5:429–440. [PubMed: 15173822]
6. Laoukili J, et al. FoxM1 is required for execution of the mitotic programme and chromosome stability. Nature Cell Biol 2005;7:126–136. [PubMed: 15654331]
7. Major ML, Lepe R, Costa RH. Forkhead box M1B transcriptional activity requires binding of Cdk-cyclin complexes for phosphorylation-dependent recruitment of p300/CBP coactivators. Mol. Cell Biol 2004;24:2649–2661. [PubMed: 15024056]
8. Wang IC, et al. Forkhead box M1 regulates the transcriptional network of genes essential for mitotic progression and genes encoding the SCF (Skp2-Cks1) ubiquitin ligase. Mol. Cell Biol 2005;25:10875–10894. [PubMed: 16314512]
9. Anderson M, et al. Plo1(+) regulates gene transcription at the M-G(1) interval during the fission yeast mitotic cell cycle. EMBO J 2002;21:5745–5755. [PubMed: 12411492]
10. Cho RJ, et al. A genome-wide transcriptional analysis of the mitotic cell cycle. Mol. Cell 1998;2:65–73. [PubMed: 9702192]
11. Darieva Z, et al. Polo kinase controls cell-cycle-dependent transcription by targeting a coactivator protein. Nature 2006;444:494–498. [PubMed: 17122856]
12. Watanabe N, et al. M-phase kinases induce phospho-dependent ubiquitination of somatic Wee1 by SCFbeta-TrCP. Proc. Natl Acad. Sci. USA 2004;101:4419–4424. [PubMed: 15070733]
13. Toyoshima-Morimoto F, Taniguchi E, Nishida E. Plk1 promotes nuclear translocation of human Cdc25C during prophase. EMBO Rep 2002;3:341–348. [PubMed: 11897663]
14. Casenghi M, et al. Polo-like kinase 1 regulates Nlp, a centrosome protein involved in microtubule nucleation. Dev. Cell 2003;5:113–125. [PubMed: 12852856]
15. Hansen DV, Loktev AV, Ban KH, Jackson PK. Plk1 regulates activation of the anaphase promoting complex by phosphorylating and triggering SCFβTrCP-dependent destruction of the APC Inhibitor Emi1. Mol. Biol. Cell 2004;15:5623–5634. [PubMed: 15469984]
16. Neef R, et al. Phosphorylation of mitotic kinesin-like protein 2 by polo-like kinase 1 is required for cytokinesis. J. Cell Biol 2003;162:863–875. [PubMed: 12939256]
17. Zhou T, Aumais JP, Liu X, Yu-Lee LY, Erikson RL. A role for Plk1 phosphorylation of NudC in cytokinesis. Dev. Cell 2003;5:127–138. [PubMed: 12852857]
18. Wonsey DR, Follettie MT. Loss of the forkhead transcription factor FoxM1 causes centrosome amplification and mitotic catastrophe. Cancer Res 2005;65:5181–5189. [PubMed: 15958562]
19. Leung TW, et al. Over-expression of FoxM1 stimulates cyclin B1 expression. FEBS Lett 2001;507:59–66. [PubMed: 11682060]
20. Jang YJ, Lin CY, Ma S, Erikson RL. Functional studies on the role of the C-terminal domain of mammalian polo-like kinase. Proc. Natl Acad. Sci. USA 2002;99:1984–1989. [PubMed: 11854496]
21. Seong YS, et al. A spindle checkpoint arrest and a cytokinesis failure by the dominant-negative polo-box domain of Plk1 in U-2 OS cells. J. Biol. Chem 2002;277:32282–32293. [PubMed: 12034729]
22. Elia AE, et al. The molecular basis for phosphodependent substrate targeting and regulation of Plks by the Polo-box domain. Cell 2003;115:83–95. [PubMed: 14532005]
23. Lowery DM, Lim D, Yaffe MB. Structure and function of Polo-like kinases. Oncogene 2005;24:248–259. [PubMed: 15640840]
24. Elia AE, Cantley LC, Yaffe MB. Proteomic screen finds pSer/pThr-binding domain localizing Plk1 to mitotic substrates. Science 2003;299:1228–1231. [PubMed: 12595692]
25. Gumireddy K, et al. ON01910, a non-ATP-competitive small molecule inhibitor of Plk1, is a potent anticancer agent. Cancer Cell 2005;7:275–286. [PubMed: 15766665]
26. Yarm FR. Plk phosphorylation regulates the microtubule-stabilizing protein TCTP. Mol. Cell Biol 2002;22:6209–6221. [PubMed: 12167714]
27. Nakajima H, Toyoshima-Morimoto F, Taniguchi E, Nishida E. Identification of a consensus motif for Plk (Polo-like kinase) phosphorylation reveals Myt1 as a Plk1 substrate. J. Biol. Chem 2003;278:25277–25280. [PubMed: 12738781]
28. Sherr CJ, McCormick F. The RB and p53 pathways in cancer. Cancer Cell 2002;2:103–112. [PubMed: 12204530]

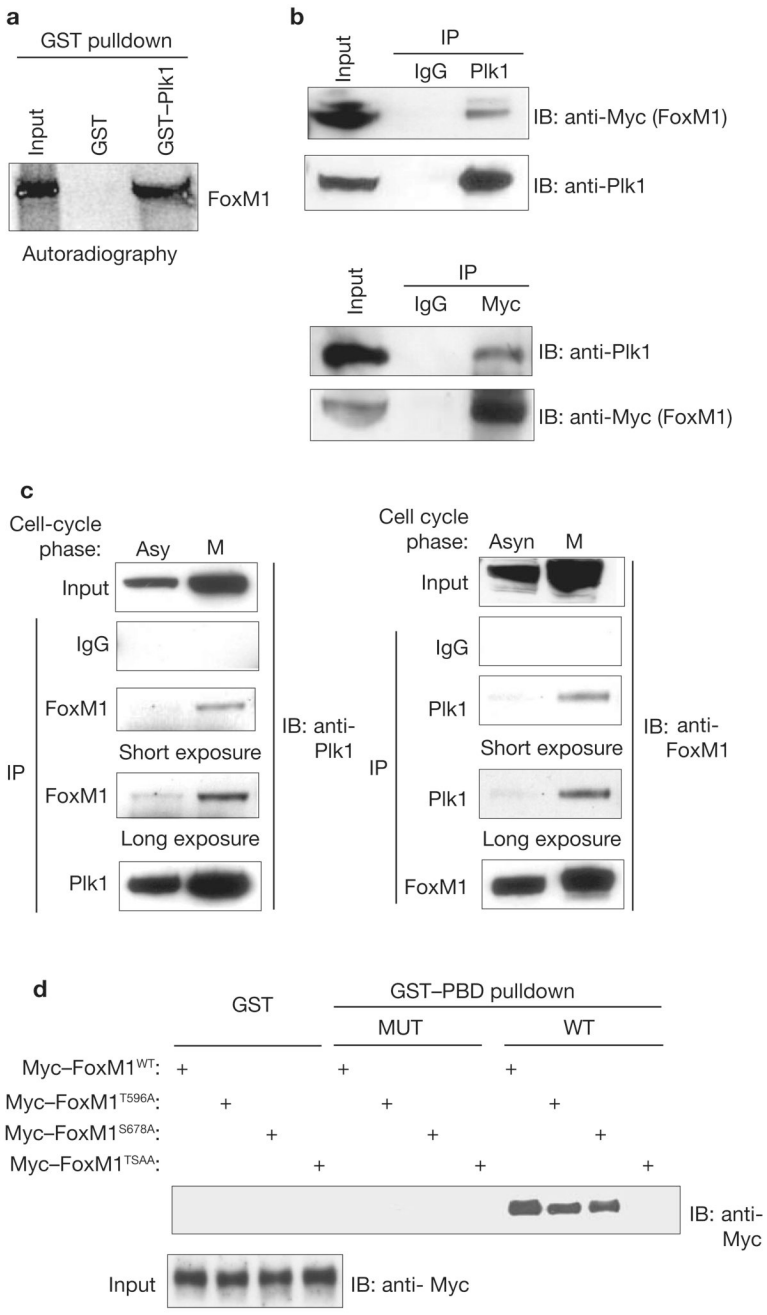

Figure 1.

Plk1 interacts with FoxM1 *in vitro* and *in vivo*. (**a**) *In vitro* translated FoxM1 was used in a pulldown assay with either GST or GST–Plk1 immobilized on agarose beads. (**b**) 293T cells were transfected with constructs encoding Myc-tagged FoxM1 and co-immunoprecipitation assays were performed. (**c**) Cell extracts were prepared from asynchronous (Asyn) or mitotic (M) HeLa cells and subjected to immunoprecipitation using anti-FoxM1 or anti-Plk1 antibodies. Immunoblotting was carried out using anti-Plk1 or anti-FoxM1 antibodies. (**d**) 293T cells were transfected with plasmids encoding Myc-tagged wild-type ($FoxM1^{WT}$) or FoxM1 mutants ($FoxM1^{T595A}$, $FoxM1^{S678A}$ or $FoxM1^{TSAA}$). Cell lysates were subjected to pulldown assays using beads coated with GST–Plk1 PBD wild-type (WT) or a mutant (MUT)

deficient in phosphopeptide binding. Beads coated with GST alone were used as a negative control. Full scans of blots in **a–d** are shown in Supplementary Information, Fig. S5.

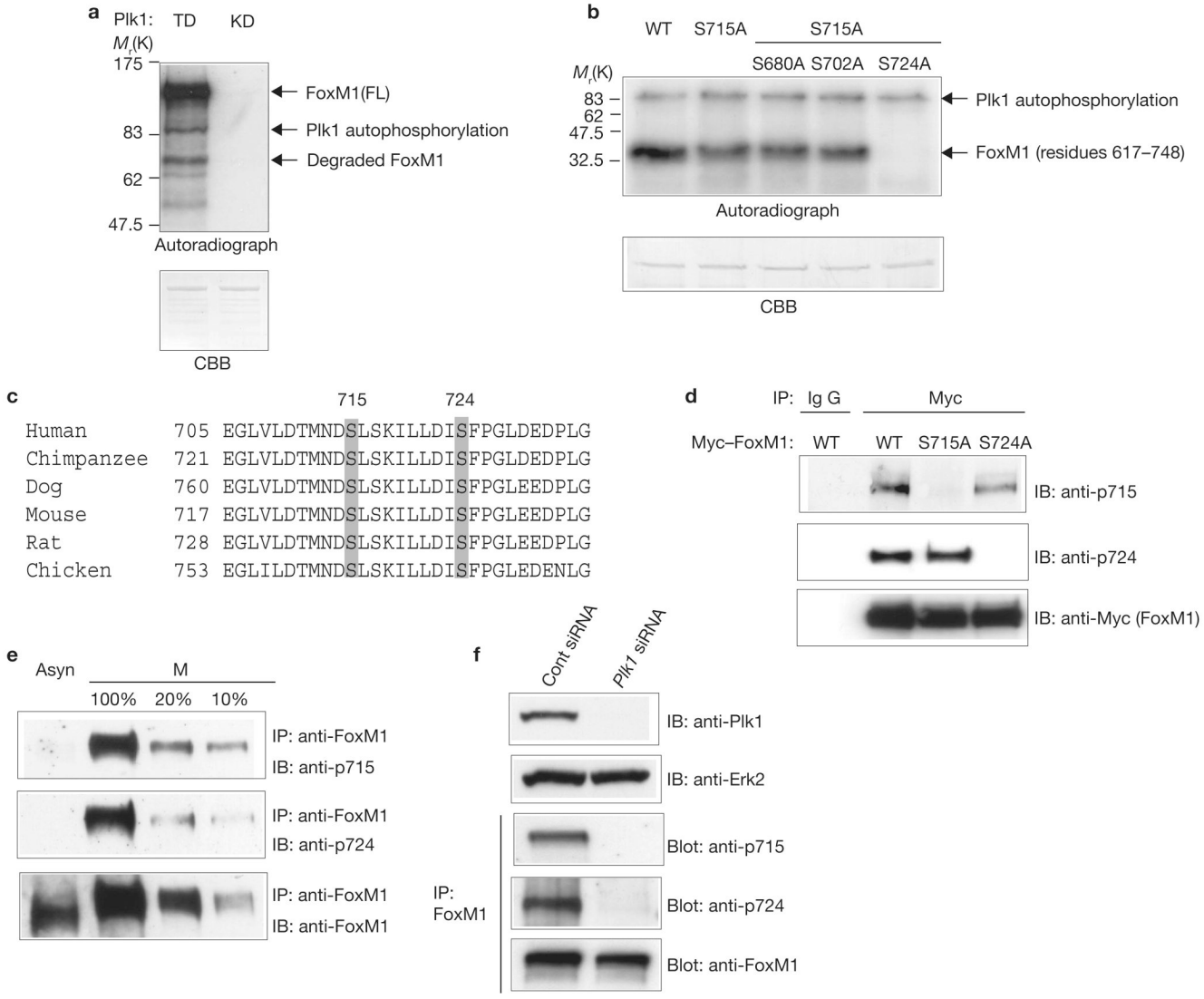

Figure 2.

Plk1 phosphorylates FoxM1 *in vitro* and *in vivo*. (**a**) Bacterially expressed FoxM1 was subjected to *in vitro* kinase assays. Kinases used were constitutively active human Plk1 (TD) or a kinase-defective (KD) mutant of Plk1 purified from insect cells. Loading controls are shown in the bottom panel (CBB, Coomassie blue staining). (**b**) FoxM1 transactivation domain mutants were used as substrates for *in vitro* kinase assays, similarly to that described in **a**. (**c**) Comparison of the extreme C-terminal sequence of FoxM1 orthologues. The two major phosphorylation sites, Ser 715 and Ser 724, are highlighted. (**d**) 293T cells were transfected with plasmids encoding Myc-tagged $FoxM1^{WT}$ or $FoxM1^{S715A}$ and $FoxM1^{S724A}$ mutants. Ectopically expressed FoxM1 proteins were immunoprecipitated with an anti-Myc antibody and immunoblotted with anti-p715, anti-p724 or anti-FoxM1 antibodies. (**e**) Endogenous FoxM1 was immunoprecipitated from cell lysates prepared from asynchronous (Asyn) or mitotic (M) HeLa cells. The phosphorylation status of FoxM1 was examined by western blotting using anti-p715 and anti-p724 antibodies. (**f**) HeLa cells were transfected with *Plk1*-specific siRNA (*Plk1*) or nonspecific control siRNA (Cont) against firefly luciferase. Nocodazole was added to arrest cells at mitosis 32 h after transfection. After a further 16 h of

incubation, endogenous FoxM1 was immunoprecipitated and western blots were performed. Erk2 was included as a loading control. Full scans of blots in **d–f** are shown in Supplementary Information, Fig. S5.

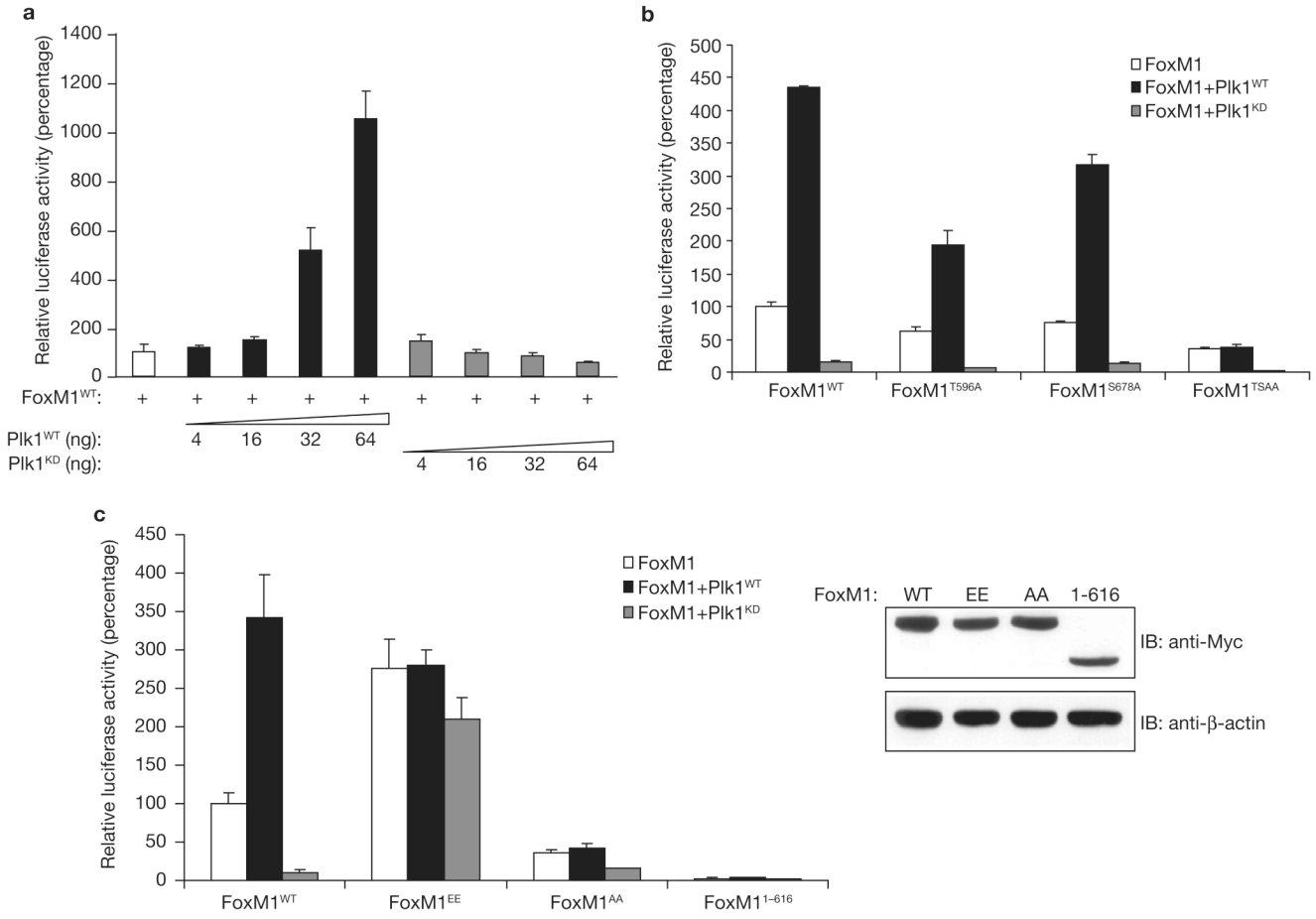

Figure 3.

Plk1 activates FoxM1 transcriptional activity. (**a**) U2OS cells were transiently transfected with the reporter plasmid 6×*FoxM1* TATA–luciferase plasmid encoding wild-type FoxM1, and increasing amounts of plasmids encoding for either wild-type Plk1 ($Plk1^{WT}$) or kinase-defective Plk1 ($Plk1^{KD}$). (**b**) U2OS cells were transfected with the FoxM1 reporter plasmid and plasmids encoding for either wild-type FoxM1 ($FoxM1^{WT}$) or FoxM1 mutants ($FoxM1^{T596A}$, $FoxM1^{S678A}$ or $FoxM1^{TSAA}$) together with plasmids encoding for WT or KD Plk1. (**c**) U2OS cells were transfected with the FoxM1 reporter plasmid and plasmids encoding for either Myc-tagged $FoxM1^{WT}$, FoxM1 mutants ($FoxM1^{AA}$, $FoxM1^{EE}$), or a FoxM1 TAD-deletion mutant ($FoxM1^{1–616}$) together with Plk1 WT or KD expression vectors. Luciferase activities were measured 24 h after transfection. Luciferase levels were normalized to *Renilla* luciferase activity. The data are expressed as a percentage of wildtype FoxM1 activity (mean ± s.d. of three separate experiments in triplicate). Expression levels of exogenous wild-type and mutant FoxM1 were monitored by western blot analysis.

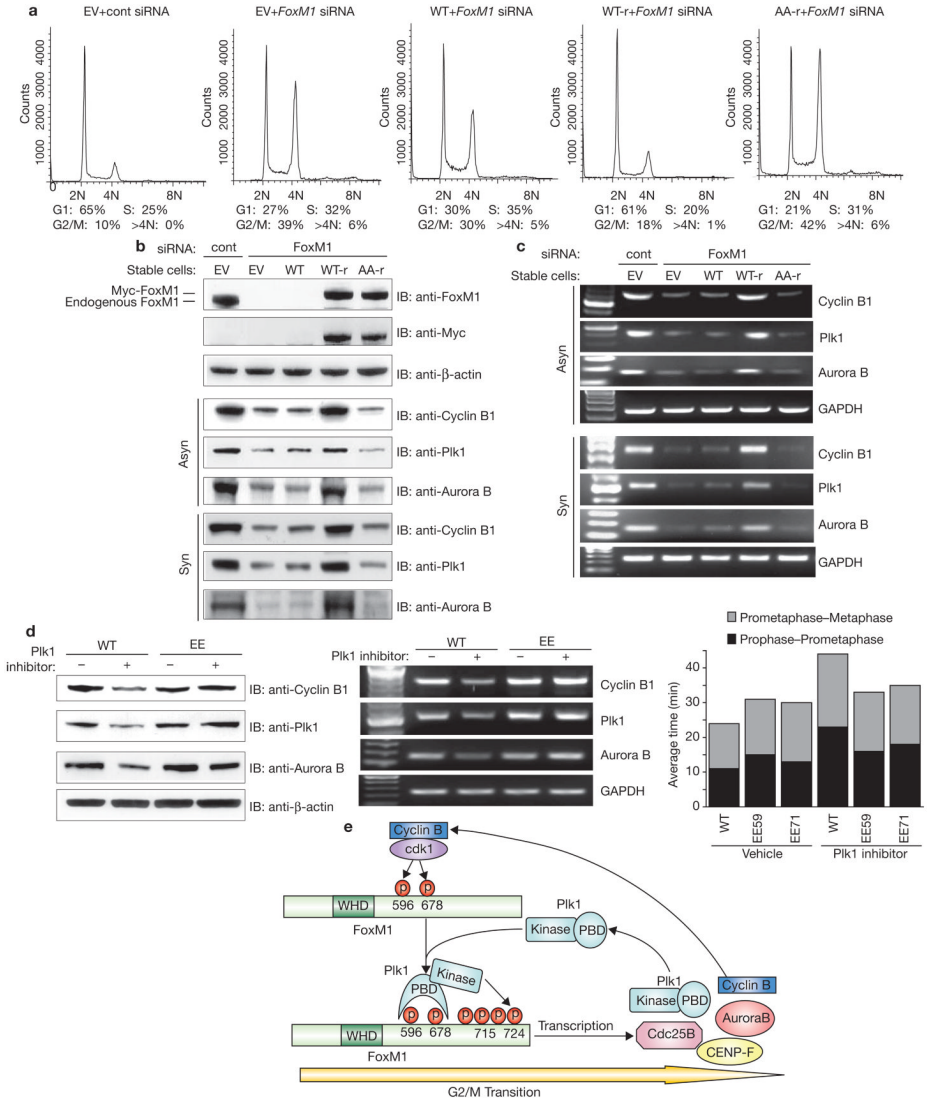

Figure 4.

Plk1-dependent phosphorylation of FoxM1 is required for expression of the G2/M transcriptional programme and orderly mitotic progression *in vivo*. U2OS cells stably expressing empty vector (EV), or Myc-tagged FoxM1^{WT}, siRNA-resistant wild-type FoxM1 (WT-r) or siRNA-resistant S715A;S724A mutant FoxM1 (AA-r) were transfected with *FoxM1*-specific siRNA (FoxM1) or nonspecific control siRNA (cont). (**a**) Cell-cycle distributions were determined by flow cytometry analysis 72 h after transfection. (**b**) Lysates were collected 72 h after transfection and analysed by immunoblotting (Asyn). Alternatively, cells were synchronized by nocodazole treatment for 16 h before collection (Syn). Blotting for β-actin was used as a loading control. (**c**) Total RNA was extracted 48 h after transfection and subjected to RT–PCR analysis. RT–PCR for *GAPDH* was used as an internal control. (**d**) U2OS cells stably expressing FoxM1 wildtype (WT) or phosphomimetic mutants (EE) were synchronized by thymidine block and released in the presence of either vehicle (DMSO) or ON01910 (250 nM) for 9 h. Cells were subjected to RT–PCR and immunoblotting analysis or monitored by time-lapse imaging. Two independent stable clones (EE59 and EE71) were used in time-lapse microscopy. Inhibition of Plk1 activity led to 91% of wild-type FoxM1-

expressing cells arresting at prometaphase, which was partially rescued in phosphomimetic FoxM1-expressing cells (EE59, 67%, EE71, 68%). For cells that eventually progressed through mitosis, the duration of each stage of mitosis was recorded and average time was plotted in the bar chart. (**e**) Model for Plk1-mediated regulation of FoxM1 transcriptional activity at G2/M transition.