Cdk1 phosphorylates SPAT-1/Bora to trigger PLK-1 activation and drive mitotic entry in C. elegans embryos

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Introduction

Regulation of cell cycle duration is crucial for the development of multicellular organisms. The first embryonic division of the Caenorhabditis elegans (C.e.) embryo is asymmetric and generates two blastomeres (AB and P1) of different sizes and developmental potentials that divide asynchronously (Deppe et al., 1978; Sulston et al., 1983). This asynchrony of cell division can be easily monitored by time-lapse differential interference contrast (DIC) microscopy, which makes this embryo an ideal system to study the pathways regulating the timing of mitotic entry in a developmental context.

Mitosis is triggered by the concerted action of several conserved mitotic kinases, notably the CyclinB/Cdk1 and members of Polo-like families (Archambault and Glover, 2009). C.e. PLK-1, similar to all Polo-like kinase family members, is characterized by an N-terminal kinase domain and a C-terminal noncatalytic region containing two tandem Polo boxes (Polo box domain [PBD]), which recognize phosphorylated peptides (Polo docking sites; Elia et al., 2003a,b; Nishi et al., 2008).

Like other family members, PLK-1 localizes at centrosomes and at kinetochores, but it is also enriched in the anterior cytoplasm in one-cell embryos and therefore becomes preferentially segregated to the AB cell in two-cell embryos (Chase et al., 2000; Budirahardja and Gönczy, 2008; Nishi et al., 2008; Rivers et al., 2008). The higher levels of PLK-1 in AB compared with P1 promote faster nuclear import of CDC-25.1, the Cdk1–activating phosphatase, and trigger earlier mitotic entry in AB compared with P1 (Rivers et al., 2008).

A regulator of Plk1 activity is the conserved protein Bora. Bora was originally identified in Drosophila melanogaster and was shown to activate Aurora A (Hutterer et al., 2006). In human cells (Macárek et al., 2008; Seki et al., 2008) and in C.e. (Noatynska et al., 2010), Bora/Suppressor of Par-Two 1 (SPAT-1) was reported to function as a Plk1 activator. In human cells, Bora binds Plk1 and enhances Aurora A–mediated T-loop activation and drive mitotic entry in C. elegans embryos

Abbreviations used in this paper: C.e., C. elegans; DIC, differential interference contrast; dsRNA, double-stranded RNA; H.s., Homo sapiens; LC, liquid chromatography; MBP, Maltose-binding protein; MS, mass spectrometry; NEBD, nuclear envelope breakdown; PBD, Polo box domain; Sf9, Spodoptera frugiperda 9; SPAT-1, Suppressor of Par-Two 1; WT, wild type.

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phosphorylation of Plk1, which is critical for full Plk1 activation (Macůrek et al., 2008; Seki et al., 2008).

Although SPAT-1/Bora is required for Plk1 activation, the regulation of the interaction between SPAT-1/Bora and Plk1 is unclear (Bruinsma et al., 2012). Here, we find that CDK-1 phosphorylates SPAT-1 to regulate its interaction with PLK-1 and to enhance Aurora A–mediated T-loop phosphorylation of PLK-1 in vitro. Mutations that mimic the nonphosphorylatable forms of SPAT-1 strongly impair mitotic entry time of early C.e. embryos. We also show that the phosphorylation of human Bora by Cdk1 similarly enhances T-loop phosphorylation of human Plk1 by Aurora A. Overall, our results suggest a model in which SPAT-1/Bora is part of a positive feedback loop that coordinates PLK-1 and CDK-1 activation for timely mitotic entry.

Results and discussion

Phosphorylation of SPAT-1 depends on Cdk1

SPAT-1 is a phosphoprotein modified at multiple residues observed as slower migrating bands on 1D gel separation (Fig. 1 A, lanes 1 and 2; Noatynska et al., 2010). Given that SPAT-1 is a PLK-1 substrate (Noatynska et al., 2010), these forms could correspond to species phosphorylated by PLK-1. However, SPAT-1–phosphorylated forms accumulated in plk-1 temperature-sensitive mutant embryos and in PLK-1–depleted embryos (Fig. 1 A, lanes 5 and 6; Noatynska et al., 2010), indicating that at least another kinase phosphorylates SPAT-1 in vivo.

Phosphorylation of SPAT-1 by CDK-1 promotes its interaction with PLK-1

Phosphorylated forms of SPAT-1 are enriched in PLK-1 immunoprecipitates (Fig. 1 C, lane 3; Noatynska et al., 2010) and a reduced fraction of phospho–SPAT-1 was recovered in the PLK-1 immunoprecipitation from embryos with partial cdk-1(RNAi) (Fig. 1 C, compare lanes 3 and 4, the pSPAT-1/PLK-1 ratio...
decreased from 1 in control to 0.2 in cdk-1(RNAi) embryos), suggesting that CDK-1 might phosphorylate SPAT-1 to promote its interaction with PLK-1. To test this hypothesis, we developed an in vitro binding assay (Fig. 1 D) in which full-length Strep–SPAT-1 produced in insect Spodoptera frugiperda 9 (Sf9) cells was immobilized on Strep-Tactin Sepharose beads. CyclinB/Cdk1 was added in the presence or absence of ATP (±ATP) to trigger SPAT-1 phosphorylation. CyclinB/Cdk1 kinase was washed away, and immobilized SPAT-1 or phospho (p)–SPAT-1 was incubated with PLK-1 for pull-down experiments. As shown in Fig. 1 D, pSPAT-1 (incubated with CyclinB/Cdk1 and ATP) pulled down PLK-1 (Fig. 1 D, lane 3) as compared with the nonphosphorylated version (Fig. 1 D, lane 4, incubated with the kinase but without ATP) consistent with the in vivo finding that hyperphosphorylated forms of SPAT-1 coimmunoprecipitate with PLK-1. These results indicate that CyclinB/Cdk1-dependent phosphorylation of SPAT-1 promotes its interaction with PLK-1.

**CDK-1 phosphorylates SPAT-1 at multiple sites**

To identify the residues phosphorylated by CDK-1, we subjected SPAT-1, phosphorylated in vitro by human CyclinB/Cdk1, to tandem mass spectrometry (MS; liquid chromatography [LC]–MS/MS) analysis and identified 13 (S/T)p residues (S36, T57, T78, S119, S190, T229, S251, T328, S368, T456, T465, S504, and T518; Fig. 2, A and B; and Table S2). A nonphosphorylatable SPAT-1 13A mutant (with all S and T of the (S/T)p sites mutated to alanine) displayed a significantly dampened phosphorylation by CyclinB/Cdk1 (Fig. 2 C), indicating that these 13 residues are indeed the major CyclinB/Cdk1 phosphorylation sites. MS analysis of endogenous SPAT-1 or GFP::SPAT-1 immunoprecipitated from WT embryonic extracts confirmed that SPAT-1 is phosphorylated in vivo on at least 7 (S36, T57, S190, T229, S251, T328, and S504) out of the 13 residues that are phosphorylated by Cdk1 in vitro (Materials and methods; Fig. 2 B and Table S2).

We next asked whether SPAT-1 phosphorylation on these sites regulates its interaction with PLK-1. We coexpressed Strep–SPAT-1 WT or Strep–SPAT-1 13A mutant with 6x(His)–PLK-1 in insect Sf9 cells and performed a Strep pull-down experiments. SPAT-1 WT, but not the SPAT-1 13A version, coprecipitated PLK-1 (Fig. 2 D, compare lanes 4 and 5). We corroborated these observations using our in vitro assay and found that the SPAT-1 13A mutant was not able to bind PLK-1 in a Cdk1-dependent manner in vitro (Fig. 2 E, lanes 1 and 2). This lack of interaction is not caused by the overall misfolding
nonphosphorylated MBP-Bora was able to stimulate human Plk1 phosphorylation on the T loop by Aurora A in vitro (Fig. 3 E). However, this effect was enhanced when MBP-Bora was pre-phosphorylated by Cdk1 (Fig. 3 E), indicating that Bora phosphorylation by Cdk1 stimulates human Plk1 phosphorylation by Aurora A.

We next tested whether phosphorylation of C. elegans SPAT-1 by Cdk1 similarly stimulates C. elegans PLK-1 phosphorylation by Aurora A. As shown in Fig. 3 F, the activity of Aurora A toward C. elegans PLK-1 was not enhanced by the addition of the MBP control or nonphosphorylated MBP–SPAT-1. However, addition of MBP–SPAT-1 phosphorylated by Cdk1 stimulated C. elegans PLK-1 phosphorylation at T194 by Aurora A (Fig. 3 F), whereas the nonphosphorylatable SPAT-1 13A mutant was defective in promoting PLK-1 phosphorylation by Aurora A (Fig. 3 G). We conclude that phosphorylation of both C. elegans SPAT-1 and human Bora by Cdk1 stimulates phosphorylation of PLK-1 at the T loop by Aurora A.

Contribution of CDK-1 phosphorylation sites to SPAT-1 function in vivo
To analyze the role of SPAT-1 phosphorylation in vivo, we generated lines expressing GFP::spat-1 RNAi-resistant transgenes (hereafter, GFP::spat-1WT) and aggregation of the SPAT-1 13A mutated protein because SPAT-1 13A was fully soluble in our extraction conditions and did not elute in the void volume in gel filtration experiments (Fig. 2 F). Collectively, these results indicate that SPAT-1 phosphorylation by CDK-1 regulates its interaction with PLK-1.

**SPAT-1 phosphorylation stimulates Aurora A-dependent phosphorylation of PLK-1 on its activator T loop**

In human cells, Bora has been suggested to trigger Plk1 activation by promoting its phosphorylation on the T loop (residue T210) by Aurora A at the G2/M cell cycle transition (Macúrek et al., 2008; Seki et al., 2008). At this transition, Bora is highly phosphorylated (Chan et al., 2008; Seki et al., 2008), raising the hypothesis that Bora phosphorylation might stimulate Aurora A activity toward Plk1.

To test this, we developed an in vitro assay that specifically monitors the activity of Aurora A toward *Homo sapiens* (H.s.) Plk1/C.e. PLK-1 in the presence of *H. sapiens* Bora/C.e. SPAT-1, phosphorylated or not by CyclinB/Cdk1 (Fig. 3 A and S1). We used a phosphospecific antibody that recognizes phosphorylated Threonine 210 of *H. sapiens* Plk1 and the equivalent phosphorylated residue in C.e. PLK-1 (T194; Fig. 3, B–D). Consistent with previous studies (Macúrek et al., 2008; Seki et al., 2008), nonphosphorylated MBP-Bora was able to stimulate human Plk1 phosphorylation on the T loop by Aurora A in vitro (Fig. 3 E). However, this effect was enhanced when MBP-Bora was prephosphorylated by Cdk1 (Fig. 3 E), indicating that Bora phosphorylation by Cdk1 stimulates human Plk1 phosphorylation by Aurora A.

We next tested whether phosphorylation of *C. elegans* SPAT-1 by Cdk1 similarly stimulates *C. elegans* PLK-1 phosphorylation by Aurora A. As shown in Fig. 3 F, the activity of Aurora A toward *C. elegans* PLK-1 was not enhanced by the addition of the MBP control or nonphosphorylated MBP–SPAT-1. However, addition of MBP–SPAT-1 phosphorylated by Cdk1 stimulated *C. elegans* PLK-1 phosphorylation at T194 by Aurora A (Fig. 3 F), whereas the nonphosphorylatable SPAT-1 13A mutant was defective in promoting PLK-1 phosphorylation by Aurora A (Fig. 3 G). We conclude that phosphorylation of both *C. elegans* SPAT-1 and human Bora by Cdk1 stimulates phosphorylation of PLK-1 at the T loop by Aurora A.

Contribution of CDK-1 phosphorylation sites to SPAT-1 function in vivo

To analyze the role of SPAT-1 phosphorylation in vivo, we generated lines expressing GFP::spat-1 RNAi-resistant transgenes (hereafter, GFP::spat-1WT) and with different combination of
Figure 4. **SPAT-1 phosphorylation at the N-terminal part is essential in vivo.** (A) Summary table presenting the SPAT-1 mutants analyzed in vivo. Sp/Tp sites mutated into alanine residues are highlighted in dark gray. (B) Schematic of the approach used to analyze the role of SPAT-1 phosphorylation in vivo. Transgenic C. elegans lines expressing RNAi-resistant SPAT-1 (SPAT-1\^R) WT (0A) or mutated on different phosphorylation sites were generated. Endogenous *spat-1* was depleted by injecting dsRNA in the different lines to measure embryonic lethality and embryonic cell cycle length in AB and P1 blastomeres. (C) Western blot analysis of embryos expressing GFP::spat-1\^R transgenes using SPAT-1 (top) and Tubulin (loading control; bottom) antibodies. (D) Graph showing the percentage of lethality of embryos (Emb.) from animals of the indicated genotype after RNAi-mediated depletion of endogenous *spat-1*. Error bars indicate 95% confidence limits. (E, top) Schematic of the first and second cell divisions of early C. elegans embryos. DNA is in blue, and black arrows show nuclear envelope breakdown (NEBD) in AB and P1 blastomeres, which is apparent by loss of the smooth line corresponding to the nuclear envelope. The mean elapsed time in seconds between ingress of the cytokinesis furrow (black arrowheads) in P0 (P0 cytokinesis) and NEBD in AB (NEBD-AB; left) or P1 (NEBD-P1; right) was determined and plotted in embryos of the indicated genotypes (shown at the bottom). Cell cycle timing was similarly measured in *plk-1* (or689) embryos for comparison. Error bars indicate 95% confidence limits, and asterisks denote a statistically significant difference: ****, \(P < 0.0001\); ***, \(P = 0.0001\); *, \(P < 0.05\) using Student’s *t* test; ns denotes a lack of statistically significant difference.
The phosphorylation of S251 residue might promote SPAT-1 binding, suggesting that S190 is important (Fig. 4 D). Consistently, a transgene expressing GFP::spat-1S190A similarly resulted in 22% embryonic lethality when endogenous SPAT-1 was depleted (Fig. 4 D). Likewise a GFP::spat-1S19A mutant transgene results in 21% embryonic lethality in the absence of endogenous SPAT-1, indicating that both S119 and S190 contribute to SPAT-1 function. We were not able to obtain transgenic lines expressing GFP::spat-1R mutated only at T229 (mutation present in the 7AN), which prevented us from analyzing the contribution of this site to the phenotype.

We then investigated whether the embryonic lethality observed in the various mutants correlated with cell cycle timing defects. We depleted SPAT-1 by RNAi in the strains expressing GFP::spat-1 WT and mutant transgenes and measured cell cycle length by time-lapse DIC microscopy. Consistently with the lethality data, we found that mitotic entry was only weakly delayed in embryos expressing GFP::spat-16AC and 4AN (Fig. 4 E). On the contrary, mitotic entry was strongly delayed, both in AB and P1 blastomeres, in embryos expressing GFP::spat-113A or GFP::spat-17AN protein (Fig. 4 E and Table S3). However, these embryos were not as delayed as spat-1(RNAi) embryos, indicating that these mutated versions retain some function in vivo. The cell cycle was also significantly delayed in embryos expressing 5AN (4AN + S190A), S119A, or S190A transgenes (Table S3).

Collectively, these results indicate that SPAT-1 phosphorylation is critical to regulate mitotic entry and that the S119 and S190 residues are important for SPAT-1 function. In the future, it will be important to study the phosphorylation kinetics of these two residues and investigate the regulatory role of multiple phosphorylation. Studies on the inactivation of the Wee1 kinase by Cdk1 in Xenopus laevis egg extracts have shown that this is an ultrasensitive system as a result of the competition between two sets of Cdk1 phosphorylation sites in Wee-1 (Kim and Ferrell, 2007). Given the robustness of cell division timing regulation in the C.e. embryo, it is tempting to speculate that a similar competition between Cdk1 phosphorylation sites may occur in SPAT-1.

In conclusion, we show that Cdk1 phosphorylates SPAT-1 to promote its interaction with PLK-1 and to trigger PLK-1 activation by Aurora A. We suggest that this is crucial to control the time of mitotic entry in the early embryo. Our results are consistent with a model in which SPAT-1 is part of a positive feedback loop that coordinates PLK-1 and Cdk1 activation (Fig. 5). Furthermore, we show that Bora phosphorylation by Cdk1 also stimulates Plk1 phosphorylation by Aurora A on its activator T loop in vitro, suggesting that the regulatory mechanism connecting Cdk1 and Plk1 activation is evolutionarily conserved.
Materials and methods

Nematode strains and culture conditions
C.e. strains were cultured and maintained using standard procedures (Brenner, 1974). Strains of the following genotypes were used: N2 and plc-1(or983ts) (provided by B. Bowerman, University of Oregon, Eugene, OR; O’Rourke et al., 2010). Transgenic GFP::spat-1 WT and mutant lines were generated by microinjection of unc-119(ed3) mutant worms as previously described (Proctor et al., 2001) with the following modifications: 20–50 µg DNA was used per transformation. Each 100-mm worm plate was bombarded twice with the same DNA construct using the Biolistic Particle Delivery System (Bio-Rad Laboratories) with a Hepta adapter. Strains generated in this study were ZU139 (unc-119(ed3) III; [pGM897; pie-1::GFP::spat-1 WT; pie-1 3’UTR + unc-119(+)]); ZU197 ([unc-119(ed3) III; [pGM871; pie-1::GFP::spat-1 WT; pie-1 3’UTR + unc-119(+)]); [pLFP98; unc-119(ed3) III]; [pL1247; pie-1::GFP::spat-1 R (S36A, T57A, T78A, S119A, S190A, T229A, S251A, T328A, S368A, T456A, T465A, S504A, T518A); pie-1 3’UTR + unc-119(+)]); [pL1242; pie-1::GFP::spat-1 R (S36A, T57A, T78A, S119A, S190A, T229A, S251A; pie-1 3’UTR + unc-119(+)]); [pie-1::GFP::spat-1 R (S36A, T57A, T78A, S119A, S190A, T229A, S251A); pie-1 3’UTR + unc-119(+)]; [pie-1::GFP::spat-1 R 5AN]]; WLP518 ([unc-119(ed3) III]; [pLM1049; pie-1::GFP::spat-1 R (S36A, T57A, T78A, S119A, S251A; pie-1 3’UTR + unc-119(+)]); [pie-1::GFP::spat-1 R 7A)]; WLP412 ([unc-119(ed3) III]; [pL1285; pie-1::GFP::spat-1 R (T328A S368A T465A S504A T518A); pie-1 3’UTR + unc-119(+)]; [pie-1::GFP::spat-1 R 6AC]); ZU236 ([unc-119(ed3) III]; [pLM1036; pie-1::GFP::spat-1 R (S36A, T57A, T78A, S190A, S251A; pie-1 3’UTR + unc-119(+)]); [pie-1::GFP::spat-1 R 4AN]); ZU243 ([unc-119(ed3) III]; [pMG1040; pie-1::GFP::spat-1 R (S36A, T57A, T78A, S190A, S251A; pie-1 3’UTR + unc-119(+)]); [pie-1::GFP::spat-1 R 7AN]); ZU246 ([unc-119(ed3) III]; [pL1285T; pie-1::GFP::spat-1 R 4AN]; [pie-1::GFP::spat-1 R 7AN]); ZU250 ([unc-119(ed3) III]; [pie-1::GFP::spat-1 R 4AN]; [pie-1::GFP::spat-1 R 7AN]).

DNA manipulation and molecular genetics
The list of plasmids used in this study is provided in Table S1. Plasmid constructions designed for yeast two-hybrid experiments, to produce recombinant MBP–SPAT-1 proteins and to generate lines expressing GFP::SPAT-1 fusion proteins, were generated by Gateway cloning according to the manufacturer’s instructions (Invitrogen). Yeast two-hybrid analysis of the GFP::SPAT-1 transgenes, we consistently checked expression of the transgenes in each embryo that we recorded to ensure that the phenotype is not a result of loss of expression.

For worm imaging, gravid adults were incubated in M9 buffer containing 20 mM levamisole and mounted on a 2% agarose pad. Pictures were acquired on an inverted microscope (Axio Observer; Carl Zeiss) using a 4× objective, equipped with DIC optics and a color camera (AxioCam HRc; Carl Zeiss). Mosaics were reconstructed by the Zen pro 2012 hardware (Carl Zeiss) using default settings.

Yeast two-hybrid analysis
Yeast two-hybrid analysis was performed using a GAL4-based system (Gateway) in the Pl69-4A yeast strain (MATa trpl-1001 leu2-3, 112 ura3-52 his3-200 gal4[10] gal80[0] lys2::GAL1-HIS3 GAL12-ADE2 met2::GAL7- lacZ; Jones et al., 1996) expressing as bait the PLK-1 PBD (C-terminal 309 amino acids of PLK-1; Noataynska et al., 2010) and full-length SPAT-1 WT, SPAT-1 S251A, or S251D as preys. Transformed clones hosting the prey bait–positive interaction were selected on selective medium lacking leucine, tryptophan, and histidine and containing 25 mM 3AT drug (3-amino-1,2,4-triazole; Sigma-Aldrich). Nine independent colonies were tested for each interaction.

Protein purification and coimmunoprecipitation
MBP and MBP–SPAT-1 fusion proteins were expressed in Escherichia coli BL21 DE3 pLysS (Invitrogen) strain and purified on amylose resin according to the manufacturer’s instructions (New England Biolabs, Inc.). In brief, MBP–SPAT-1 expression was induced by addition of 1 mM isopropyl β-D-thiogalactopyranoside (Sigma-Aldrich) to 1 liter of Terrific Broth culture of BL21 DE3 pLysS before incubation at 37°C for 2 h. After centrifugation, the bacteria were resuspended in 20 ml lysis buffer (20 mM Hepes, pH 7.4, 200 mM NaCl, 2 mM DTT, and 1 mM EDTA) and lysed with a homogenizer (EmulsiFlex; Avestin). The soluble portion of the lysate was subjected to amylose resin resin. The resin was washed with 10 volumes of lysis buffer, and bound proteins were eluted in lysis buffer containing 20 mM maltose (Sigma-Aldrich).

Recombinant proteins expressed in insect S9 cells were produced with the Bacto-Bac Bacteriophage Expression System according to the manufacturer’s instructions (Invitrogen). Bacteriophages expressing the C.e. PBD in fusion with GST were a gift from T. Hyman (Max Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany; Decker et al., 2011). To produce C.e. 6xHis–PLK-1 WT and C.e. 6xHis–PLK-1 T194A, insect S9 cells were infected with appropriate bacteriophages and then lysed in lysis buffer (PBS, pH 7.2, 250 mM NaCl, 30 mM imidazole, and protease and phosphatase inhibitors [Roche]) by passing the cell suspension 30 times through a 21-gauge syringe needle. The lysate was clarified by centrifugation for 10 min at 16,000 g, and the supernatant was injected on Hitrap Chelating HP column loaded with nickel sulfate (GE Healthcare). Proteins were eluted by an imidazole gradient using a fast protein LC Äkta System (GE Healthcare). Most purified elution fractions were pooled, diluted volume to volume in the lysis buffer without imidazole and containing 50% glycerol, concentrated on a centrifugal concentrator (Vivaspin V15R12; Vivaproducs), flash frozen in liquid nitrogen, and stored at −80°C.

For proteins in Figs. 1 D and 2 C (−), 5.3 × 10^6 insect S9 cells were plated in 6-well plates and infected or coinfected with the appropriate combination of viruses and incubated at 27°C for 72 h. Cells were then lysed in 300 µl lysis buffer (PBS, pH 7.2, 250 mM NaCl, and protease inhibitors [Roche]) by passing the cell suspension 30 times through a 21-gauge syringe needle. The lysate was clarified by centrifugation for 10 min at 16,000 g, and the supernatant was incubated with 50 µl Strep-Tactin Sepharose beads or 25 µl glutathione–Sepharose 4B beads (GE Healthcare) for 1 h at 4°C. The beads were washed five times and used for subsequent experiments. In the case of GST-tagged proteins, elution was performed with 3x Laemmli buffer (Fig. S2).

For the PLK-1 pull-down assay presented in Figs. 1 D and 2 E, 6xHis–PLK-1 WT and C.e. 6xHis–PLK-1 T194A insect S9 cells were infected with appropriate bacteriophages and then lysed in lysis buffer (PBS, pH 7.2, 250 mM NaCl, 30 mM imidazole, and protease and phosphatase inhibitors [Roche]) by passing the cell suspension 30 times through a 21-gauge syringe needle. The lysate was clarified by centrifugation for 10 min at 16,000 g, and the supernatant was incubated with 50 µl Strep-Tactin Sepharose beads or 25 µl glutathione–Sepharose 4B beads (GE Healthcare) for 1 h at 4°C. The beads were washed five times and used for subsequent experiments. In the case of GST-tagged proteins, elution was performed with 3x Laemmli buffer (Fig. S2).

For the PLK-1 pull-down assay presented in Figs. 1 D and 2 E, an extract of insect S9 cells expressing 6xHis–PLK-1 was incubated on Strep-Tactin Sepharose beads coupled to Strep–SPAT-1 WT or mutant, dephosphorylated by λ phosphatase (see Phosphatase treatment section), and rephosphorylated or not rephosphorylated by H.s. CyclinB/Cdk1 (see Kinase assays section) during 1 h at 4°C. The beads were then washed five times, and proteins were eluted with 5 µl desthiobiotin and analyzed by SDS-PAGE and Western blotting.

For coimmunoprecipitation experiments of PLK-1 from embryonic extracts, worms were grown in liquid culture, and embryos were harvested from gravid worms by bleaching (500 mM NaOH and 15% bleach). Packed embryos were frozen in liquid nitrogen and stored at −80°C.
were then thawed, resuspended in immunoprecipitation buffer (100 mM KCl, 50 mM Tris, pH 7.5, 1 mM MgCl₂, 1 mM DTT, 5% glycerol, 0.05% NP-40, 1 mM EDTA, and protease and phosphatase inhibitor cocktail [Roche]), and frozen in liquid nitrogen. The embryos were ground on dry ice using a mortar and pestle. The protein homogenate was thawed on ice and centrifuged at 13,000 rpm for 30 min at 4°C. 450 μg protein was incubated for 4 h at 1 h with 10 ng of either PLK1 or IgG antibody. Next, 15 μl protein A beads was added to each tube for 1 h at 4°C. The NaCl buffer was washed away, and the protein A beads were washed five times with immunoprecipitation buffer and boiled with SDS sample buffer.

Gel filtration analysis of SPAT-1

Strep–SPAT-1 WT and Strep–SPAT-1 13A proteins produced in insect Sf9 cells were captured on Strep-Tactin Sepharose beads, phosphorylated with or without protease and phosphatase inhibitors (New England Biolabs, Inc.), and eluted in PBS buffer, pH 7.2, and 250 mM NaCl containing 5 mM dithiothreitol. Eluted proteins were centrifuged for 10 min at 16,000 g and 10 μl was injected on a Superdex 200 10/300 GL column (GE Healthcare) equilibrated in PBS, pH 7.2, and 250 mM NaCl at a flow rate of 0.5 ml/min on a fast protein LC Akta System. The column was calibrated using a Gel filtration calibration kit (Sigma-Aldrich), which contains a mixture of five different proteins (669-kD thyroglobulin, 443-kD apoferritin, 200-kD chymotrypsinogen, 150-kD alcohol dehydrogenase, and 29-kD carbonic anhydrase).

Biochemical assays

Phosphatase treatment.

Dephosphorylation of immobilized Strep–SPAT-1 WT or mutants on Strep-Tactin Sepharose beads was performed for 2 h in kinase buffer (50 mM Tris, pH 7.5, 15 mM MgCl₂, 2 mM EGTA, 1 mM DTT, and protease and phosphatase inhibitors [Roche]) with 20 U phosphatase buffer supplemented with MnCl₂ and 400 U WT or mutants on Strep-Tactin Sepharose beads (Figs. 1 D and 2 E). Subsequent steps were performed in the presence of phosphatase inhibitors containing either 300 ng full-length MBP–SPAT-1 or MBP (Figs. 1 B, 3, and S1) or immobilized Strep–SPAT-1 on Strep-Tactin Sepharose beads (Figs. 1 D and 2 C and E). Reactions were initiated by adding a mix of 0.2 mM ATP and, in the specific cases of Figs. 1 B and 2 C, 5 μCi [γ-32P]ATP (PerkinElmer), during 40 min at 30°C. For these experiments, samples were boiled in Laemmli buffer 3x and visualized by Coomassie blue staining.

Kinase assays.

CyclinB/Cdk1-dependent kinase assays were performed in CdK1 kinase buffer (50 mM Hepes, pH 7.6, 10 mM MgCl₂, 1 mM DTT, and protease and phosphatase inhibitors [Roche]) with 20 U Hs. CyclinB/Cdk1 (New England Biolabs, Inc.) in a final volume of 30 μl containing either 300 ng full-length MBP–SPAT-1 or MBP (Figs. 1 B, 3, and S1) or immobilized Strep–SPAT-1 on Strep-Tactin Sepharose beads (Figs. 1 D and 2 C and E). Reactions were initiated by adding a mix of 0.2 mM ATP and, in the specific cases of Figs. 1 B and 2 C, 5 μCi [γ-32P]ATP (PerkinElmer), during 40 min at 30°C. For these experiments, samples were boiled in Laemmli buffer 3x and visualized by Coomassie blue staining.

Western blotting and antibodies

Western blot analysis was performed using standard procedures (Sambrook et al., 1989). For PLK1 antibodies, the C-terminal 180 bp of PLK1 was cloned into pDEST51 using Gateway technology (Life Technologies), and the purified GST fusion protein was injected into rabbits. The serum was purified on a MBP–PLK1 column and used at 1:1,000 for the described experiments. Other antibodies include SPAT-1 (rabbit; 1:10,000; Noatynska et al., 2010), MBP (1:1,000; mouse; New England Biolabs, Inc.), Tubulin 1:1,000 (mouse, DM1A; Sigma-Aldrich), Actin 1:1,000; mouse, clone C4; MP Biomedicals), Plk1 pT210 (1:1,000; rabbit; Cell Signaling Technology), and phospho-Ser [CdK] substrate (1:1,000; rabbit; Cell Signaling Technology). His-tagged anti-mouse and anti-rabbit antibodies (Sigma-Aldrich) were used at 1:3,000, and the signal was detected with chemiluminescence (Thermo Fisher Scientific).

Identification of SPAT-1 phosphorylation sites by LC-MS/MS

For the analysis of SPAT-1 phosphorylated in vitro (Fig. 2 A), the protein mixture containing MBP–SPAT-1 phosphorylated by CyclinB/Cdk1 was digested overnight at 37°C using sequencing grade trypsin (12.5 μg/ml; Promega). The peptides were separated in 20 μl of 25% acetonitrile/0.1% NH₄HCO₃. Digests were desalted by a LIQ Velos Orbitrap (Thermo Fisher Scientific) coupled to an Easy nano-LC Proxeon system (Thermo Fisher Scientific). An Easy column Proxeon C18 (2 cm, 100 μm inner diameter, and 120 A) was used for peptide preconcentration and an Easy Column Proxeon C18 (10 cm, 75 μm inner diameter, and 120 A) for peptide separation. Chromatographic separation of the peptides was performed with the following parameters: 300 nL/min flow, gradient ranging from 95% solvent A (water–0.1% formic acid) to 25% B (100% acetonitrile and 0.1% formic acid) in 20 min and then to 45% B in 40 min and finally to 80% B in 10 min. Peptides were analyzed on the Orbitrap in a second scan event. The maximum ion accumulation times were set to 100 ms for MS acquisition and 50 ms for MS/MS acquisition. MS/MS data were acquired in a data-dependent mode in which the 20 most intense precursor ions were isolated, with a dynamic exclusion of 20 s, an exclusion mass width of 10 ppm, an exclusion list size of 300, and a repeat duration of 30 s.

For the analysis of SPAT-1 phosphorylation sites in vivo (Fig. 2 A), SPAT-1 or GFP–SPAT-1 was captured from embryo extracts using SPAT-1 antibodies (Noatynska et al., 2010) or GFP nanobodies (Rothbauer et al., 2008; ChromoTek), respectively. The beads carrying the immune complexes were washed with binding buffer and then rinsed with 100 μl of 25-mmol/liter NH₄HCO₃. Proteins on beads were digested overnight at 37°C using sequencing grade trypsin (12.5 μg/ml) in 20 μl of 25-mmol/liter NH₄HCO₃. The same chromatographic, MS conditions, and data analysis parameters as described in the previous paragraph were used.

Improvement of phosphorylation detection from in vivo phosphorylated SPAT-1.

To improve the sensitivity for the phosphorylated peptides of SPAT-1 found in vitro in mutant animals.

Data processing of MS analysis.

Data were processed with Proteome Discoverer 1.4 software (Thermo Fisher Scientific) coupled to an in-house Mascot search engine (version 2.1.1, Matrix Science). The mass tolerance of fragment ions was set to 7 ppm for precursor and 0.5 D for fragment ions. The following modifications were used in variable modifications: oxidation (M) and phosphorylations (STY). Phosphorylation localizations were evaluated by the Phospho-RS 3.1 algorithm (Taus et al., 2011). The maximum number of missed cleavages was limited to 2 for trypsin digestion. MS–MS data were searched against the NCBI-nr (nonredundant) database. The false discovery rate was calculated using the support vector machine–based algorithm Percolator with a 5% false discovery rate in relaxed mode and 1% q-value in stringent mode was used. A threshold of 1% was chosen for this rate.

Online supplemental material

Fig. S1 documents control experiments relevant to Fig. 3 (phosphorylation of PKL1/PKL1 is strictly dependent on Aurora A). Fig. S2 shows the lack of interaction of the Polo docking site mutant (SPAT-1 S251A) with the PBD of PKL1 but the ability of this mutant and the double (SPAT-1 S250A and S251A) mutant to rescue the sterility of spat-1(s3061) mutant animals. Table S1 is the list of plasmids used in this study. Table S2 shows the MS data as described in Fig. 2 A. A 37°C timed run online as an Excel (Microsoft) file. Table S3 documents the statistics relative to Fig. 4 and is provided online as an Excel file. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201408064/DC1.
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