Polo-like kinase 4 kinase activity limits centrosome overduplication by autoregulating its own stability

Andrew J. Holland,1,2 Weijie Lan,1,2 Sherry Niessen,3,4 Heather Hoover,3,4 and Don W. Cleveland1,2

1Ludwig Institute for Cancer Research and 2Department of Cellular and Molecular Medicine, University of California, San Diego, La Jolla, CA 92093
3The Skaggs Institute for Chemical Biology and 4Department of Chemical Physiology, The Center for Physiological Proteomics, The Scripps Research Institute, La Jolla, CA 92037

Accurate control of the number of centrosomes, the major microtubule-organizing centers of animal cells, is critical for the maintenance of genome integrity. Abnormalities in centrosome number can promote errors in spindle formation that lead to subsequent chromosome missegregation, and extra centrosomes are found in many cancers. Centrosomes are comprised of a pair of centrioles surrounded by amorphous pericentriolar material, and centrosome duplication is controlled by centriole replication. Polo-like kinase 4 (Plk4) plays a key role in initiating centriole duplication, and overexpression of Plk4 promotes centriole overduplication and the formation of extra centrosomes. Using chemical genetics, we show that kinase-active Plk4 is inherently unstable and targeted for degradation. Plk4 is shown to multiply self-phosphorylate within a 24-amino acid phosphodegron. Phosphorylation of multiple sites is required for Plk4 instability, indicating a requirement for a threshold level of Plk4 kinase activity to promote its own destruction. We propose that kinase-mediated, autoregulated instability of Plk4 self-limits Plk4 activity so as to prevent centrosome amplification.

Introduction

Centrosomes are the major microtubule-organizing centers of animal cells and play an important role in organizing the interphase cytoskeleton and poles of the mitotic spindle (Nigg, 2002). A single centrosome is comprised of a pair of centrioles surrounded by an amorphous pericentriolar material, and centrosome duplication is controlled by centriole replication (Loncarek and Khodjakov, 2009). In dividing cells, centrioles duplicate once per cell cycle adjacent to preexisting centrioles. Errors in this process lead to the production of an abnormal centrosome number that can promote errors in spindle formation and subsequent chromosome missegregation (Ganem et al., 2009; Silkwort et al., 2009). Moreover, abnormalities in centrosome number are often observed in cancer cells; and thus, accurate control of centrosome number is critical for the maintenance of genome integrity (Nigg, 2006; Holland and Cleveland, 2009).

Centriole duplication is regulated by the conserved protein kinase Polo-like kinase 4 (Plk4; also known as SAK). Overexpression of Plk4 overrides the mechanism that limits centriole duplication, leading to multiple rounds of centriole duplication within a single cell cycle (Bettencourt-Dias et al., 2005; Habedank et al., 2005; Peel et al., 2007; Basto et al., 2008). Plk4 is aberrantly expressed in human colorectal and liver cancer (Macmillan et al., 2001; Ko et al., 2005). Furthermore, Plk4−/− mice develop spontaneous liver and lung tumors (Ko et al., 2005), whereas overexpression of Plk4 promotes centrosome amplification and tumorigenesis in flies (Basto et al., 2008; Castellanos et al., 2008). Therefore, Plk4 activity must be precisely regulated to accurately control centriole duplication and prevent transformation.

Plk4 is a low abundance protein with a short cellular half-life (Fode et al., 1996). Plk4 is ubiquitinated and degraded by the 26S proteasome (Fode et al., 1996; Cunha-Ferreira et al., 2009; Korzeniewski et al., 2009; Rogers et al., 2009), and inhibiting proteasome function promotes centriole overduplication in a Plk4-dependent manner (Duensing et al., 2007). The SKP1–CUL1–F Box (SCF) complex is an E3 ubiquitin ligase comprised of three core subunits (Skp1, Cullin 1, and Rbx1) and

Supplemental Material can be found at:
http://jcb.rupress.org/content/suppl/2010/01/25/jcb.200911102.DC1.html
Plk4 (Fig. 1A) were stably expressed in a human cell line (DLD-1) in which accurate chromosome segregation maintains a pseudodiploid karyotype (Lengauer et al., 1997). To facilitate direct comparison, WT and kinase-dead (KD) Plk4-EYFP transgenes were integrated at a predefined genomic locus in the host cell line using flippase (Flp) recombination target (FRT)/Flp–mediated recombination. Plk4-EYFP expression was under tight tetracycline regulation, allowing temporal control of Plk4 kinase-induced centriole biogenesis (Fig. S1, A and C). Previous work has shown that overexpressed Plk4 KD can increase centrosome numbers in cycling populations of cells, whereas Plk4WT promotes centrosome amplification in S phase–arrested cells (Habedanck et al., 2005), suggesting dual pathways (one kinase dependent and another that is kinase independent) for Plk4 stimulation of centrosome duplication. Indeed, 48 h after induction of Plk4 WT, 60% of asynchronously growing cells showed evidence of centriole amplification, whereas induction

Results and discussion

To determine how mammalian Plk4 levels are established, EYFP-tagged wild-type (WT) and kinase-inactive (D154A) mouse Plk4 (Fig. 1A) were stably expressed in a human cell line (DLD-1) in which accurate chromosome segregation maintains a pseudodiploid karyotype (Lengauer et al., 1997). To facilitate direct comparison, WT and kinase-dead (KD) Plk4-EYFP transgenes were integrated at a predefined genomic locus in the host cell line using flippase (Flp) recombination target (FRT)/Flp–mediated recombination. Plk4-EYFP expression was under tight tetracycline regulation, allowing temporal control of Plk4 kinase-induced centriole biogenesis (Fig. S1, A and C). Previous work has shown that overexpressed Plk4KD can increase centrosome numbers in cycling populations of cells, whereas Plk4WT promotes centrosome amplification in S phase–arrested cells (Habedanck et al., 2005), suggesting dual pathways (one kinase dependent and another that is kinase independent) for Plk4 stimulation of centrosome duplication. Indeed, 48 h after induction of Plk4WT, 60% of asynchronously growing cells showed evidence of centriole amplification, whereas induction

Figure 1. Plk4 kinase activity destabilizes the protein. (A) Schematic of mouse Plk4, showing the location of the kinase domain, the Polo-box domain (PBD), and the cryptic Polo-box domain (Swallow et al., 2005). The locations of the AS and KD mutations are also shown. (B) Immunoblot showing the expression level of WT and KD Plk4-EYFP transgenes. (C) Titration showing the relative expression level of Plk4WT and Plk4KD. (D) Immunoblot showing the expression levels of Plk4WT, Plk4AS, and Plk4KD in the presence of DMSO or 1NM-PP1. (E) Immunofluorescence images acquired 48 h after Plk4-EYFP was induced with tetracycline. Cells were grown in DMSO or 1NM-PP1. Insets depict an enlargement of centrioles. Red, SAS-6; green, Plk4-EYFP; blue, DNA. Bars, 10 µm. (F) Graph quantifying the proportion of cells with more than four centrioles 48 h after expression of Plk4-EYFP was induced. Cells were grown in the presence of either DMSO or 1NM-PP1. Bars represent the mean of at least three independent experiments with >300 cells per condition. Error bars represent the SEM.
of Plk4KD caused centriole amplification in a 2.5-fold smaller proportion (23%) of cells (Fig. 1, E and F; and Fig. S1 B).

Surprisingly, the total level of Plk4KD protein was >10-fold higher than that of Plk4WT (Fig. 1, B and C), and the level of Plk4KD at the centriole was >45-fold higher than that of Plk4WT (Fig. S1, D and E), implicating kinase activity of Plk4 as a determinant of its stability. To test this hypothesis, we created an ATP analogue-sensitive (AS) mutant of Plk4 (Bishop et al., 2000). Plk4AS possesses a point mutation at a conserved gatekeeper residue (L89G) that enlarges the ATP-binding pocket and allows the kinase to be selectively inhibited by the bulky ATP analogue 1NM-PP1 (Fig. 1 A). To characterize the Plk4AS allele, we expressed and purified recombinant WT, AS, and KD GST-Plk4 and tested the ability of the kinase to phosphorylate histone H1 in vitro. GST-Plk4WT phosphorylated itself (in cis or trans) and the substrate histone H1, whereas GST-Plk4KD lacked detectable activity (Fig. S2, A and B). In comparison with GST-Plk4WT, GST-Plk4AS exhibited an ∼2.5 reduction in phosphorylation of itself and an ∼10-fold reduction in phosphorylation of the histone H1 (Fig. S2, A and B, which are reductions comparable with that observed with a similar Plk1AS allele that was shown to compensate for the loss of endogenous Plk1 in cells (Burkard et al., 2007). As expected, in vitro 1NM-PP1 inhibited the activity of GST-Plk4AS but not GST-Plk4WT (Fig. S2 C). Therefore, we created stable cell lines expressing Plk4AS-EYFP and examined Plk4’s stability and ability to promote centriole amplification in the presence or absence of the 1NM-PP1. In the absence of drugs Plk4AS was expressed at a low level that promoted centriole overduplication in 64% of cells, which is similar to that observed with Plk4WT (Fig. 1, D–F). In contrast, addition of 1NM-PP1 caused a dramatic increase in the abundance of Plk4AS and reduced centriole overduplication to the level observed with Plk4KD (Fig. 1, D–F). This suggests that the activity of Plk4AS can be inhibited by 1NM-PP1 in vivo and confirms that Plk4 kinase activity regulates its own stability.

The 26 S proteasome inhibitor MG132 was added to Plk4WT and Plk4KD-expressing cells to determine whether Plk4 kinase activity promotes proteasome-mediated Plk4 destruction. Plk4WT but not Plk4KD was substantially stabilized by MG132 treatment (Fig. 2 A). To determine whether kinase-active Plk4 is capable of destabilizing Plk4AS in trans, we induced expression of Plk4AS-EYFP or Plk4AS-EYFP in cells constitutively expressing Plk4KD-Myc. Expression of Plk4AS-EYFP, but not Plk4KD-EYFP, reduced the stability of Plk4KD-Myc, demonstrating that Plk4 can promote self-destruction through intermolecular phosphorylation (Fig. 2 B).

Our data support a model in which autophosphorylation triggers Plk4’s destruction to self-limit kinase activity. Plk4 possesses a conserved SCF-β-TrCP phosphodegron, and previous studies in Drosophila cells have implicated a role of this two-phosphosite phosphodegron in regulating Plk4’s stability (Cunha-Ferreira et al., 2009; Rogers et al., 2009). Therefore, we considered the possibility that autophosphorylation (in cis or trans) of the β-TrCP phosphodegron in promoting Plk4’s destruction to self-limit kinase activity. Plk4 possesses a conserved SCF-β-TrCP destruction motif only plays a minor role in regulating the stability of Plk4 in these human cells.

The ubiquitination and degradation of Plk4AA (Fig. 2, D and F) suggested that phosphorylation of additional sites is required to promote Plk4’s destruction. Therefore, mass spectrometry (MS) was used to map additional in vitro autophosphorylation sites on GST-Plk4WT (Fig. S3 A). A densely
His-Plk4\textsuperscript{24} was reduced by about a third (36%) compared with that of His-Plk4\textsuperscript{WT} (Fig. 3 C). Together, these data demonstrate that Plk4 multiply autophosphorylates a 24-aa region (aa 282–305) and that this region is not required for kinase activity in vitro.

We next tested whether aa 282–305 were involved in regulating Plk4 stability in cells. Importantly, deletion of aa 282–305 (Plk4\textsuperscript{24}) dramatically stabilized Plk4 (Fig. 3 D). Plk4\textsuperscript{24} induced centriole overduplication in \( \approx 75\% \) of cells, and such cells contained a mean of \( \approx 17 \) centrioles per cell compared with about eight centrioles for cells expressing Plk4 WT (Fig. 3, E–G). This suggests that Plk4 multiply autophosphorylates a 24-aa phosphodegron (282–305) that is required to destabilize kinase-active Plk4.

To determine how phosphorylation contributes to the instability of kinase-active Plk4, we created several Plk4 mutants that were defective in phosphorylation at specific sites within aa 282–305 (Fig. 4 A): Plk4\textsuperscript{AA} contains two mutations in the phosphorylated region containing 13 phosphorylatable aa within a 24-aa stretch (282–305) was identified (Fig. S3, B and C). This region contains the previously identified \( \beta\)-TrCP two-phosphosite phosphodegron (Cunha-Ferreira et al., 2009; Rogers et al., 2009) and is part of a putative PEST domain (aa 272–311) previously proposed to destabilize human Plk4 (Fig. 3 A; Yamashita et al., 2001). Nine peptides within the 282–305 region were identified with multiple (up to four) phosphorylations, albeit the exact positions of those sites could not be identified with certainty because of the close packing of phosphorylatable amino acids (Fig. S3, B and C).

GST-Plk4WT readily phosphorylated His-Plk4\textsuperscript{KD} (aa 1–416), but His-Plk4\textsuperscript{24} (lacking aa 282–305) accumulated 42% less phosphorylation (Fig. 3 B). Furthermore, although kinase-active His-Plk4\textsuperscript{WT} and His-Plk4\textsuperscript{24} phosphorylated histone H1 with similar efficiency, autophosphorylation of His-Plk4\textsuperscript{24} was reduced by about a third (36%) compared with that of His-Plk4\textsuperscript{WT} (Fig. 3 C). Together, these data demonstrate that Plk4 multiply autophosphorylates a 24-aa region (aa 282–305) and that this region is not required for kinase activity in vitro.
we favor the interpretation that Plk4 directly promotes its own destruction through autophosphorylation of a 13-site multiphosphodegron (Fig. 4 E). The multiphosphodegron possesses a conserved β-TrCP–binding site previously implicated in regulating the stability of Drosophila Plk4 (Cunha-Ferreira et al., 2009; Rogers et al., 2009). A mutant of mouse Plk4 unable to be phosphorylated in the β-TrCP–binding site (Plk4AA) exhibited dramatically reduced β-TrCP binding; however, Plk4 AA was ubiquitinated and only modestly stabilized in cells. In contrast, deletion of the entire 24-aa multiphosphodegron or mutation of all 13 phosphorylation sites in this region dramatically stabilized Plk4, suggesting that autophosphorylated Plk4 interacts with additional SCF F box proteins or alternative E3 ubiquitin ligases that are required for degradation. Surprisingly, we found that repression of β-TrCP stabilized Plk4 AA, indicating that SCFβ-TrCP still contributes to the destruction of this mutant (Fig. S3 D). Although we suspect that this arises from an indirect effect of β-TrCP depletion, it is possible that the small amounts of β-TrCP associated with Plk4 AA still play a role in targeting the protein for destruction.

Together, our results show that Plk4’s kinase activity autoregulates Plk4 protein stability. Our data do not exclude the possibility that Plk4 activity regulates stability through additional proteins; for example, Plk4-induced activation of an unknown kinase may regulate Plk4 stability. However, we demonstrate that Plk4 is capable of autophosphorylating a region crucial for the kinase activity-dependent degradation of Plk4. Therefore, we favor the interpretation that Plk4 directly promotes its own destruction through autophosphorylation of a 13-site multiphosphodegron (Fig. 4 E). The multiphosphodegron possesses a conserved β-TrCP–binding site previously implicated in regulating the stability of Drosophila Plk4 (Cunha-Ferreira et al., 2009; Rogers et al., 2009). A mutant of mouse Plk4 unable to be phosphorylated in the β-TrCP–binding site (Plk4AA) exhibited dramatically reduced β-TrCP binding; however, Plk4 AA was ubiquitinated and only modestly stabilized in cells. In contrast, deletion of the entire 24-aa multiphosphodegron or mutation of all 13 phosphorylation sites in this region dramatically stabilized Plk4, suggesting that autophosphorylated Plk4 interacts with additional SCF F box proteins or alternative E3 ubiquitin ligases that are required for degradation. Surprisingly, we found that repression of β-TrCP stabilized Plk4 AA, indicating that SCFβ-TrCP still contributes to the destruction of this mutant (Fig. S3 D). Although we suspect that this arises from an indirect effect of β-TrCP depletion, it is possible that the small amounts of β-TrCP associated with Plk4 AA still play a role in targeting the protein for destruction.

Because Plk4 phosphorylation occurs in dynamic equilibrium with counteracting phosphatases, we speculate that a requirement for multisite phosphorylation to prime degradation is
likely to provide a temporal delay to allow Plk4 to phosphorylate critical substrates required for centriole duplication before its protein level is down-regulated. This could create a sensitive biological switch in which Plk4 can be rapidly destabilized once its activity reaches a certain threshold level. This autoregulatory feedback loop places Plk4 stability under direct control of its own activity and may form an important mechanism to limit normal centriole duplication to once per cell cycle.

Materials and methods

Constructs

The full-length mouse Plk4 open reading frame was cloned into a pcDNA5/FRT/TO-based vector (Invitrogen) modified to contain a C terminus GFP or Myc epitope tag. To create Plk4 and Plk4, two silent point mutations were introduced to create an Nhel site at aa 225–226. A fragment of mouse Plk4 (aa 223–387) was amplified by PCR and cloned into a pET30 bacterial expression vector (EMD), which contains an N-terminal 6His tag. To create GST-Plk4, the mouse Plk4 open reading frame was cloned into a pFastBac1 (Invitrogen)-based vector modified to contain an N terminus GST tag. pcDNA3 Myc–β-TrCP and pcDNA3 HA-ubiquitin plasmids were obtained from Addgene.

Cell culture

Cells were maintained at 37°C in a 5% CO2 atmosphere in DMEM containing 10% fetal calf serum (FCS; Gibco/BRL), 100 U/ml penicillin, 100 U/ml streptomycin, and 2 mM l-glutamine. DLD-1 cells were used in all experiments except in Fig. 2 (E and F), which used HEK 293 cells. Parental cells (provided by S. Taylor, The University of Manchester, Manchester, England, UK) were used for retroviral delivery as described previously (Tighe et al., 2003) using retroviral delivery as described previously (Tighe et al., 2003).

Generation of stable cell lines and sirNA treatment

RFP-tagged histone H2B (H2B-RFP) was introduced into Flp-In Trex-DLD-1 parental cells (provided by S. Taylor, The University of Manchester, Manchester, England, UK) using retroviral delivery as described previously (Shah et al., 2004). Stable integrates were selected in 2 µg/ml puromycin, and single clones were isolated using FACs (FACS憧憬ent; BD). Stable, isogenic cell lines expressing Plk4-EYFP were generated using the FRT/Flp-mediated recombination as described previously (Tighe et al., 2004). Expression of Plk4-EYFP was induced with 1 µg/ml tetracycline (Sigma-Aldrich). Flp-In Trex-DLD-1 H2B-RFP cells were used for all the experiments except in Fig. 2 (E and F). Small molecules were used at the following final concentrations: nocodazole MG132 (EMD), 20 µM; 1NM-PP1 (Stratagene, La Jolla, CA), 10 µM. For siRNA treatment, 1.5 × 105 cells were plated in a 6-well plate, and duplexed sRNAs were introduced using Oligofectamine (Invitrogen). siRNAs directed against β-TrCP (5′-GGGAAUUUGG-GGAACAU-3′) and GAPDH (5′-GGUUUACUGAUCCAAUA-3′) were purchased from Thermo Fisher Scientific. 24 h after transfection, tetra-cycline was added to induce expression of Plk4-EYFP and cells harvested and processed for immunoblotting 24 h later.

Immunoblotting and immunofluorescence

To purify Plk4-EYFP, cells were lysed in lysis buffer (10 mM Tris, pH 7.5, 0.1% Triton X-100, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 50 mM NaF, 200 mM β-glycerophosphate, 0.1 mM DTT, 200 nM microcystin, 1 mM PMSF, and 1 µM leupeptin, pepstatin, and chymostatin [LPC]), sonicated, and soluble extracts were prepared. The supernatant was incubated with beads alone or beads coupled to GFP-binding protein (GBP; Rothbauer et al., 2008). Beads were washed five times in lysis buffer, and protein complexes were analyzed by immunoblotting. For immunoblot analysis, protein samples were separated by SDS-PAGE, transferred onto nitrocellulose membranes (Bio-Rad Laboratories), and probed with antibodies 4A6, mouse anti-Myc; 1:1,000; Millipore), D11A (mouse anti-α-tubulin; 1:1,000), and YFP (mouse anti-GFP; 1:500; Roche). For immunofluorescence, cells were grown on hydrophilic acid–washed, poly-L-lysine (Sigma-Aldrich)–coated 18-mm glass coverslips and fixed in 100% ice-cold methanol for 10 min. Cells were blocked in 2.5% FBS, 200 mM glycerol, and 0.1% Triton X-100 in PBS for 1 h. Antibody incubations were conducted in the blocking solution for 1 h. DNA was detected using DAPI, and cells were mounted in Prolong Antifade (Invitrogen).

Staining was performed with antibodies SAS-6 (human anti-SAS-6; provided by A. Dammermann, Ludwig Institute for Cancer Research, San Diego, CA) and GTU-88 (mouse anti-α-tubulin; 1:250; Abcam). YFP was visualized directly using a GFP filter set, the human SAS6 antibody was directly conjugated to Cy5 (Jackson ImmunoResearch Laboratories, Inc.), and GTU-88 was stained with a secondary goat anti–mouse antibody conjugated to AMCA.

Immunofluorescence images were collected using an inverted microscope (IX-70; Olympus) with a Delovision Core system (Applied Precision) controlling an interline charge-coupled device camera (CoolSNAP; Roper Industries) driven by softWoRx software (Applied Precision). Images were collected using a 100× or 60× 1.4 NA oil objective (Olympus) at 0.2-µm z sections and subsequently deconvolved. Imaging was performed at room temperature, and maximum intensity 2D projections were generated for each image using softWoRx. With the exception of the images in Fig. S1 E, EYFP exposure times were adjusted for each Plk4-EYFP mutant. Centriole numbers were determined by counting the number of structures that stained positive for both Plk4-EYFP and SAS-6. For quantitation of EYFP, signal intensity at the centriole unconvolved 2D maximum-intensity projections was saved as unscanned 16-bit TIFF images. Signal intensity was determined using MetaMorph (MDS Analytical Technologies) by drawing a 20 × 20–pixel box and a larger 25 × 25–pixel box around the centrosome. The integrated EYFP intensity in the smaller box was calculated by subtracting the mean fluorescence intensity in the area between the two boxes (mean background) from the mean GFP intensity in the small box and multiplying by the area of the smaller box.

Protein purification

GBP was purified from Escherichia coli (strain Rosetta [DE3]) and covalently coupled to NIH-activated Sepharose 4 Fast Flow beads (GE Healthcare) at 1 mg/ml as described previously (Rothbauer et al., 2008). Recombinant His-Plk4 (aa 1–416) was transformed in E. coli (strain Rosetta [DE3]), and protein expression was induced at 16°C for 18 h with 200 nM IPTG. Bacterial pellets were suspended in lysis buffer (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 20 mM imidazole, 0.1% β-mercaptoethanol, 0.1% NP-40, 1 mM PMSF, and 1 mM LMP) and lysed by sonication after 1 mg/ml lysosome treatment on ice for 30 min. After centrifugation at 15,000 rpm (50,000 g; Sorvall) for 30 min, the supernatant was incubated with pre-washed nickel nitricacetate acid beads (QIAGEN) at 1 h at 4°C. Beads were washed extensively in wash buffer (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 20 mM imidazole, 0.1% β-mercaptoethanol, 0.1% NP-40, 1 mM PMSF, and 1 mM LMP), and protein was eluted in elution buffer (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 300 mM Imidazole, 0.1% β-mercaptoethanol, and 0.1% NP-40). Proteins were dialyzed into a final buffer containing 50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 20 mM imidazole, and 0.1% β-mercaptoethanol. Recombinant GST-Plk4 was expressed and purified from insect cells (High Five; Invitrogen) using the Bac-to-Bac expression system (Invitrogen). Infected cell pellets were suspended in lysis buffer (PBS supplemented with 2 mM MgCl2, 5 mM EDTA, 10 mM microcystin, 1 mM Na3V04 in the presence of 50 µM ATP, and 100 µCi/ml 32P) and lysed by sonication. After centrifugation at 15,000 rpm (SA-600; Sorvall) for 30 min, the supernatant was supplemented with 110 mM KCl and 0.1% Triton X-100 and incubated with glutathione Sepharose (GE Healthcare) for 1 h at 4°C. Beads were washed extensively in wash buffer (PBS supplemented with 1 mM EDTA, 0.1% Triton X-100, 100 mM KCl, 1 mM PMSF, and 1 mM LMP) and protein was eluted in elution buffer (1× PBS supplemented with 40 mM reduced glutathione, 50 mM Tris, pH 8.0, and 5 mM DTT).

In vitro kinase assays

In vitro kinase assays were performed at room temperature in 20 mM Tris, pH 7.5, 25 mM KCl, 1 mM MgCl2, 1 mM DTT, 5 mM Na3V04 in the presence of 50 µM ATP, and 100 µCi/ml 32P. 2 µg of substrate was incubated with 1 µg purified GST-Plk4 or 2 µg His-Plk4 (aa 1–416). Kinase reactions were stopped with sample buffer and analyzed by SDS-PAGE. For quantification, individual bands were excised, and the incorporation of 32P was quantitated using a scintillation counter (Beckman Coulter). For λ phosphatase treatment, GST-Plk4 was incubated with λ phosphatase (New England Biolabs, Inc.) at 30°C for 30 min.

MS sample preparation

20 µg trichloroacetic acid–precipitated GST-Plk4W7 was dissolved in 8 M urea and 100 mM Tris, pH 8.0, with a final volume of 25 µl. The protein was reduced in 5 mM TCEP (Fluka) for 20 min at room temperature and alkylated in 10 mM iodoacetamide (Sigma-Aldrich) for 15 min in the dark. Each sample was diluted fourfold with 100 mM Tris, pH 8.0, and CaCl2.
was added to a final concentration of 1 mM. 0.8 µl trypsin (0.5 µg/µl; Promega) was added for an overnight digestion at 37°C. Samples were acidified with formic acid to a final concentration of 5% for MS analysis.

**MS analysis**

The tryptic digest was pressure loaded onto an equilibrated fused silica capillary column containing 3 cm Aqua 5u C18 (Phenomenex) packed into a 250-µm-i.d. capillary with a filtered union (UpChurch Scientific). Next, an equilibrated 100µm-i.d capillary with a 5µm pulled tip containing 10 cm of C18 material was attached to the filter union and placed in line with a quaternary HPLC (1200; Agilent Technologies). Peptides were separated with a 2-h organic gradient (Dix et al., 2008) and analyzed on an LTQ-Orbitrap as previously described (Lu et al., 2008), except one full Fourier transform mass spectrum (400–1,800 m/z; resolution of 60,000) was followed by nine data-dependent MS/MS acquired in the linear ion trap.

**Analysis of MS data**

The ms² spectra data were extracted from the raw files using the publicly available RAW Xtractor (version 1.9.1). Ms² spectra data were searched using the ProLuCID search algorithm (Xu, J.D. Venable, S. Kyu Park, D. Cociorva, B. Lu, L. Liao, J. Wohlschlegel, J. Hewel, and J.R. Yates III. 2006. Human Proteome Organisation Fifth Annual World Congress. Abstr. S271) against a custom-made database containing the longest entry form of the mouse International Protein Index database (version 3.26) associated with each Ensembl gene identifier, resulting in a total of 22,833 unique entries. Additionally, each of these entries was reversed and appended to the database for assessment of false-positive rates. In total, the search database contained 45,666 protein sequence entries (22,833 real sequences and 22,833 decoy sequences). Searches allowed for the search database contained 45,666 protein sequence entries (22,833 real sequences and 22,833 decoy sequences). Searches allowed for the search database contained 45,666 protein sequence entries (22,833 real sequences and 22,833 decoy sequences). Searches allowed for the search database contained 45,666 protein sequence entries (22,833 real sequences and 22,833 decoy sequences). Searches allowed for the search database contained 45,666 protein sequence entries (22,833 real sequences and 22,833 decoy sequences). Searches allowed for the search database contained 45,666 protein sequence entries (22,833 real sequences and 22,833 decoy sequences). Searches allowed for the search database contained 45,666 protein sequence entries (22,833 real sequences and 22,833 decoy sequences). Searches allowed for the search database contained 45,666 protein sequence entries (22,833 real sequences and 22,833 decoy sequences).

Online supplemental material

Fig. S1 shows that kinase activity destabilizes centriole-localized Plk4. Fig. S2 shows that an AS mutant of recombinant Plk4 can be inhibited with the ATP analogue 1NM-PP1. Fig. S3 shows in vitro phosphorylation analysis of recombinant GSTPlk4. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200911102/DC1.

We thank Edward Yeh for depositing pcDNA3 HA-ubiquitin and Yue Xiong for depositing pcDNA3 Myc-β-TRCP in the Addgene plasmid repository. We also thank Stephen Taylor and Alexander Dammermann for providing reagents for this study.

This work was supported by a grant (GM29513) from the National Institutes of Health to D.W. Cleveland, who receives salary support from the Macmillan, J.C., J.W. Hudson, S. Bull, J.W. Dennis, and C.J. Swallow. 2001. Control of meiotic and mitotic progression by the F box protein beta-Trcp1 and beta-Trcp2: effects on centrosome numbers and centrosome amplification. Mol. Cell. Biol. 21:4665–4672.


Figure S1. **Kinase activity destabilizes centriole-localized Plk4.** (A) Cells were cultured in the presence or absence of tetracycline, fixed, and stained for γ-tubulin. Note that expression of Plk4WT promotes centriole overduplication. (B) Representative examples of centriole amplification observed in ~23% of cells expressing Plk4KD. (A and B) Bars, 5 µm. Red, SAS-6; green, Plk4-EYFP; blue, DNA. (C) Immunoblot showing tetracycline-inducible expression of Plk4-EYFP. (D) Graph quantifying the mean fluorescence intensity of Plk4WT-EYFP and Plk4KD-EYFP at the centriole. All images were acquired at the same exposure settings. Bars represent the mean of three independent experiments with >300 cells per condition. ***, P < 0.0001 by t test. Error bar represents SEM. (E) Immunofluorescence staining of cells expressing Plk4WT and Plk4KD. EYFP images were taken at 0.1- or 1-s exposure times (Exp), and images were scaled identically. Insets depict an enlargement of centrioles. Bars, 10 µm.
**Figure S2.** An AS mutant of recombinant Plk4 can be inhibited with the ATP analogue 1NM-PP1. (A) Coomassie staining of purified recombinant WT, AS, and KD GST-Plk4. (B) GST-Plk4 kinase assay was performed using histone H1 as a substrate. Samples were taken at 0, 5, 10, 30, and 60 min, and activity was quantified by scintillation counting. Coomassie-stained gel shows the purified protein, and the autoradiogram shows the incorporation of $\gamma^{[32P]}$ATP. GST-Plk4 phosphorylates itself and histone H1. (C) GST-Plk4 kinase assays were performed using histone H1 as a substrate. Assays were performed for 60 min in the presence of various concentrations of 1NM-PP1, and activity was quantified by scintillation counting. Coomassie-stained gel shows the purified protein, and the autoradiogram shows the incorporation of $\gamma^{[32P]}$ATP. Note the kinase activity of AS but not WT Plk4 is inhibited by 1NM-PP1. C, Coomassie.

**Figure S3.** In vitro phosphorylation analysis of recombinant GST-Plk4. (A) GST-Plk4 treated with or without $\lambda$ phosphatase for 30 min. (B) Representative spectra from the ProLuCID search containing two phosphorylations whose potential sites are highlighted with asterisks. The ms² spectra display a large neutral loss (Phs), and the b- and y-ion series are colored. (C) Summary table of the Xcorr, DeltCN, PPM, and Debunker probability score of each of the nine phosphopeptides identified. Potential site of modification are highlighted with asterisks. (D) Cells were transfected with control or $\beta$-TrCP siRNA and immunoblotted to determine the stability of Plk4-EYFP transgenes. Note that repression of $\beta$-TrCP also increases the stability of Plk4 AA, which is unable to be phosphorylated in the $\beta$-TrCP–binding site.