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Authors
Hatch, Emily M
Kulukian, Anita
Holland, Andrew J
et al.

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Cep152 interacts with Plk4 and is required for centriole duplication

Emily M. Hatch, Anita Kulukian, Andrew J. Holland, Don W. Cleveland, and Tim Stearns

Department of Biology and Department of Genetics, Stanford University, Stanford, CA 94305
Ludwig Institute for Cancer Research and Department of Cellular and Molecular Medicine, University of California, San Diego, La Jolla, CA 92039

Correspondence to Tim Stearns: stearns@stanford.edu

Introduction

Centrioles are microtubule-based structures that organize the centrosome and nucleate cilia. Centrioles duplicate once per cell cycle, and duplication requires Plk4, a member of the Polo-like kinase family; however, the mechanism linking Plk4 activity and centriole formation is unknown. In this study, we show in human and frog cells that Plk4 interacts with the centrosome protein Cep152, the orthologue of Drosophila melanogaster Asterless. The interaction requires the N-terminal 217 residues of Cep152 and the crypto Polo-box of Plk4. Cep152 and Plk4 colocalize at the centriole throughout the cell cycle. Overexpression of Cep152 (1–217) mislocalizes Plk4, but both Cep152 and Plk4 are able to localize to the centriole independently of the other. Depletion of Cep152 prevents both normal centriole duplication and Plk4-induced centriole amplification and results in a failure to localize Sas6 to the centriole, an early step in duplication. Cep152 can be phosphorylated by Plk4 in vitro, suggesting that Cep152 acts with Plk4 to initiate centriole formation.

Cep152 interacts with Plk4 and is required for centriole duplication

Emily M. Hatch, Anita Kulukian, Andrew J. Holland, Don W. Cleveland, and Tim Stearns

Department of Biology and Department of Genetics, Stanford University, Stanford, CA 94305
Ludwig Institute for Cancer Research and Department of Cellular and Molecular Medicine, University of California, San Diego, La Jolla, CA 92039

Correspondence to Tim Stearns: stearns@stanford.edu

Abbreviations used in this paper: CSF, cytostatic factor; MBP, maltose-binding protein; SMC, structural maintenance of chromosomes.

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Results and discussion

We developed a *Xenopus* egg extract system to characterize the action of Plk4 in centriole duplication. *Xenopus* embryos can make thousands of centrioles in the absence of transcription or translation (Gard et al., 1990), and egg extract can assemble centrosomes (Félix et al., 1994; Stearns and Kirschner, 1994) and duplicate centrioles (Hinchcliffe et al., 1999; Lacey et al., 1999). To study the function of Plk4 in centriole assembly, we made use of the ability of egg extracts to translate input mRNA (Murray and Kirschner, 1989). mRNA encoding the *Xenopus* Plk4 orthologue, Plx4, was added to translation-competent interphase *Xenopus* egg extract. A polyclonal antibody raised against Plx4 recognized the translated protein, whereas endogenous Plx4 was only detectable when immunoprecipitated from a larger volume of egg extract (Fig. 1 A).

We first tested whether translated Plx4 could stimulate the formation of multiple centrioles around sperm centrioles added to the extract. Addition of Plx4 mRNA and sperm centrioles together to egg extract resulted in the formation of centrosomes bearing multiple centrin foci surrounding a single bright centrin focus, presumably representing the sperm centriole, within 2 h at 16°C (Fig. 1 B). We next tested whether translated Plx4 could stimulate de novo centriole formation. Addition of Plx4 mRNA alone to egg extract resulted in the formation of many centriole-like structures within 2 h at 16°C at a mean density of 2 x 10^4/µl extract (Fig. 1 B). We judged these structures to be centrioles based on three criteria: (1) they were able to organize centrosomes, as demonstrated by their ability to form microtubule asters in mitotic extract (Fig. S1 A), (2) they were labeled by antibodies against centrosomal markers including γ-tubulin, Plx4, and acetylated α-tubulin (Fig. S1 B), and (3) they were able to serve as sites for assembly of new centrin foci after extended incubation in interphase extract (Fig. S1 C). Thus, overexpression of Plx4 in *Xenopus* extracts promotes de novo centriole formation similar to that observed in *Drosophila* eggs (Peel et al., 2007; Rodrigues-Martins et al., 2007).

Based on the ability of Plx4 to generate centrioles in *Xenopus* egg extract, we sought to identify Plx4-interacting proteins from this source. For this purpose, we used a kinase-dead form of Plx4 (Plx4-D154A), as this protein might have a more sustained interaction with binding partners. Plx4-D154A was translated in egg extract and purified by affinity chromatography, and associated proteins were identified by mass spectrometry. Seven *Xenopus* proteins were identified: Cep152, Brg1, Pbrm1, Wdr33, Cad, Chd1, and Atxn2 (Fig. S1 D). Cep152 was identified by three unique peptides and was chosen for further analysis because it was previously associated with centrosome structure and function (Andersen et al., 2003; Varmark et al., 2007; Blachon et al., 2008).

We examined the localization of endogenous Cep152 and a GFP-Cep152 fusion protein in human RPE-1 cells. Antibody against endogenous Cep152 revealed that it localized to the centrosome throughout the cell cycle (Fig. 2 A). A similar distribution was observed for transiently expressed GFP-Cep152 (unpublished data). In some cases, the apparent amount of Cep152 on the two centrosomes differed (Fig. 2 A); however, this was not correlated with centriole age or presence of a primary cilium, and likely represents experimental variation. Cep152 localization in G1 phase centrosomes of U2OS cells was examined by deconvolution microscopy and found to be distinct from that of centrin, a marker of the distal end of the centriole, and γ-tubulin, a marker of the pericentriolar material (Fig. 2 B).
Carvalho-Santos et al., 2010; Hodges et al., 2010). In contrast, Leidel et al., 2005; Basto et al., 2006; Kleylein-Sohn et al., 2007; Gönczy, 2003; Dammermann et al., 2004; Matsuura et al., 2004; ally and functionally in organisms with centrioles (Leidel and CPAP/Sas-4, and Cep135/Bld10, are widely conserved structur tion, we would expect them to have a similar evolutionary distri

mother centriole.

In duplicated centriole pairs, Cep152 signal partially overlapped with that of Sas6 (Fig. 2 C), which localizes to the proximal end of new centrioles (Kleylein-Sohn et al., 2007; Strnad et al., 2007). The observed pattern of localization is most consistent with Cep152 being concentrated at the proximal end of the mother centriole.

If Plk4 and Cep152 function together in centriole duplication, we would expect them to have a similar evolutionary distribution. Several of the core centriole components, including Sas6, CPAP/Sas-4, and Cep135/Bld10, are widely conserved structurally and functionally in organisms with centrioles (Leidel and Gönczy, 2003; Dammermann et al., 2004; Matsuura et al., 2004; Leidel et al., 2005; Basto et al., 2006; Kleylein-Sohn et al., 2007; Carvalho-Santos et al., 2010; Hodges et al., 2010). In contrast, Plk4 has a more limited distribution, being present in chordates, insects, and a few other invertebrate groups, but absent from C. elegans and most ciliated single-celled eukaryotes (Carvalho-Santos et al., 2010; Hodges et al., 2010). By searching for reciprocal BLAST hits, we found that Cep152 has the same distribution as Plk4, including absence from C. elegans, which is similar to what was reported for the Drosophila orthologue Asterless (Hodges et al., 2010). We used multiple sequence alignment of vertebrate and invertebrate orthologues of Cep152 with Drosophila Asterless to identify two conserved regions in Cep152, CR1 and CR2 (Fig. S2 A). CR1 is found in Cep152 in vertebrates and invertebrates, whereas CR2 is only present in vertebrates. In addition to these two regions, Cep152 orthologues share homology in their two structural maintenance of chromosomes (SMC)-like coiled-coil domains (Fig. 3 A, SMC-A and SMC-B).

To characterize the interaction of Cep152 with Plk4, we first tested whether the interaction could be observed in human cells as in frog egg extracts. Epitope-tagged human Plk4 and Cep152 expressed in human HEK293T cells were able to interact with the other in reciprocal immunoprecipitation experiments (Fig. S2 B). By expressing deletion constructs bearing the indicated parts of Cep152 and Plk4 as epitope-tagged proteins in HEK293T cells (Fig. 3, A and B), we found that the first 217 residues of Cep152, which includes CR1, are necessary and sufficient to bind Plk4 (Fig. 3 A) and that the crypto Polo-box region of Plk4 is necessary and sufficient to bind Cep152 (Fig. 3 B). Using two differently tagged versions of Cep152, we found that Cep152 is able to interact with itself and that at least one of the SMC-like domains is necessary for interaction with full-length Cep152 (Fig. 3 A). Lastly, we found that a region including CR2 was necessary and sufficient for localization of a GFP fusion protein to the centrosome (Figs. 3 A and S2 C). To determine whether the observed interaction of Plk4 and Cep152 is also likely to be occurring at the centrosome, we examined their localization in U2OS cells. Similar, overlapping patterns of localization were observed for both epitope-tagged versions of Plk4 and Cep152 and the endogenous proteins (Fig. 3 C).

We next tested whether Cep152, like Plk4, is required for centriole duplication in mammalian cells. U2OS cells were transfected with siRNAs against Cep152, and Cep152 protein depletion was apparent starting at 48 h after transfection, reaching maximum extent at 72 h (Fig. 4 A). Centriole number was determined by centrin labeling in mitotic cells that lacked Cep152 labeling. In contrast to cells transfected with control siRNAs, we observed a stepwise decrease in centriole number over time in Cep152-depleted cells, which is consistent with a defect in centriole duplication followed by segregation in mitosis (Fig. 4, B and C). In cells that had two or more centrioles 96 h after RNAi treatment, these centrioles were single centrioles rather than pairs, suggesting that they too resulted from failure of centriole duplication followed by a failure of segregation at mitosis (Fig. 4 B). Similar results were observed with Cep152 depletion in HeLa cells (unpublished data). Both bipolar and monopolar spindles were observed in cells with a single centriole after Cep152 depletion (unpublished data), which is similar to what is observed upon depletion of Plk4 (Habedanck et al., 2005). The centriole duplication phenotype could be rescued by expressing RNAi-resistant
normally, allowing us to specifically assay the requirement of Cep152 in centriole amplification. 18 h after induction of Plk4 overexpression, 99% of control mitotic cells had the expected amplified centriole “rosettes” at the spindle poles (Habedanck et al., 2005), whereas 64% of Plk4-overexpressing, Cep152-depleted cells had only two centrioles at each pole (Fig. 4 D). These results indicate that Cep152 is required both for normal centriole duplication and for Plk4-induced centriole amplification.

GFP-Cep152, but not by GFP, indicating that the phenotype is specific to Cep152 depletion (Fig. S3).

We next tested whether Cep152 is also required for Plk4-induced centriole amplification. Plk4 overexpression was induced in RPE-1 cells that had been depleted of Cep152, and centriole number was determined by centrin labeling. Cep152 depletion was less efficient in these cells compared with U2OS cells (Figs. 4 D and 5 B), and centriole duplication occurred normally, allowing us to specifically assay the requirement of Cep152 in centriole amplification. 18 h after induction of Plk4 overexpression, 99% of control mitotic cells had the expected amplified centriole “rosettes” at the spindle poles (Habedanck et al., 2005), whereas 64% of Plk4-overexpressing, Cep152-depleted cells had only two centrioles at each pole (Fig. 4 D). These results indicate that Cep152 is required both for normal centriole duplication and for Plk4-induced centriole amplification.
Cep152 binds the Plk4 crypto Polo-box (Fig. 3 B), expression of a Cep152 mutant containing the Plk4-binding domain but lacking the centrosome localization domain (Cep152 [1–217]) might mislocalize Plk4. Consistent with this, we found that expression of Cep152 (1–217) resulted in loss of centrosomal Plk4 labeling (Fig. 5 A) and subsequent progressive loss of centrioles, which is

Based on the aforementioned results, we envisioned several possibilities for the functional relationship between Cep152 and Plk4. The first is that one protein serves to localize the other to the centrosome and that localization is required for centriole duplication. Plk4 has been shown to localize to the centrosome via the crypto Polo-box domain (Habedanck et al., 2005). Because
similar to that observed for Cep152 depletion (not depicted). Next, we directly tested whether Cep152 is required for Plk4 localization in cells depleted of Cep152. Plk4 localization to the centrosome was unchanged in U2OS cells depleted of Cep152; similarly, Cep152 localization to the centrosome was unchanged in cells depleted of Plk4 (Fig. 5 B). These results suggest that a Cep152 fragment that binds Plk4 is sufficient to outcompete one or more other factors that localize Plk4 to the centrosome but that neither protein is necessary for localization of the other.

Plk4 is required for the localization of Sas6 and other centriole assembly proteins during centriole assembly (Kleylein-Sohn et al., 2007; Strnad et al., 2007). To determine whether Cep152 is similarly required for early centriole assembly, we assayed Sas6 localization in cells depleted of Cep152 (Fig. 5 C). Control U2OS cells had one focus of Sas6 at each centriole doublet in S phase–arrested and mitotic cells (Fig. 5 C, S phase cell). In contrast, Cep152-depleted cells that had failed to duplicate centrioles lacked Sas6 on mitotic centrioles and had either a single focus of Sas6 (65%; n = 150 cells) or reduced Sas6 (25%) on S phase centrioles (Fig. 5 C). We observed the same phenotype for Plk4 depletion (unpublished data), which is similar to the previous description of the relationship between Plk4 and Sas6 localization (Kleylein-Sohn et al., 2007; Strnad et al., 2007). These results suggest that both Cep152 and Plk4 act early in the centriole assembly pathway.

We next investigated whether Plk4 can phosphorylate Cep152. GFP-tagged Cep152 isolated from human cell lysates was incubated with purified wild-type GST-Plk4 (Plk4-WT) or the kinase-dead mutant GST-Plk4 D154A (Plk4-KD) and assayed for 32P incorporation (Fig. 5 D). Plk4-WT but not Plk4-KD was able to phosphorylate itself, as previously reported (Holland et al., 2010; Sillibourne et al., 2010). Both full-length GFP-Cep152 (Fig. 5 D, left) and the GFP-Cep152 (1–217) fragment that binds to Plk4 (Fig. 5 D, middle) were phosphorylated when incubated with Plk4-WT but not with Plk4-KD. To test whether this reflected direct phosphorylation of Cep152 by Plk4, we purified Cep152 (1–217) fused to maltose-binding protein (MBP) and incubated it with GST-Plk4. MBP-Cep152 (1–217) was phosphorylated by Plk4-WT but not by Plk4-KD (Fig. 5 D, right). In all reactions, the phosphorylation was specific to the Cep152 portion of the fusion proteins, as neither GFP nor MBP alone were phosphorylated (unpublished data). Thus, Plk4 can interact directly with Cep152 and phosphorylate it in vitro. In addition, at least one phosphorylated region corresponds to the N-terminal Cep152 fragment that is required for interaction with Plk4.

We propose that Plk4 and Cep152 act together early in centriole duplication. We have shown that Cep152 is a centriolar protein that interacts with Plk4 and can be phosphorylated by it in vitro. Cep152 is required for centriole duplication, Plk4 overexpression–induced centriole amplification and, as for Plk4, for localization of Sas6 to centrioles, which is an early step in their assembly. The hypothesis that Plk4 and Cep152 act together in centriole duplication is also reinforced by their pattern of evolutionary conservation. The identification of Cep152 as a Plk4-associated factor provides a new tool for understanding the function of Plk4 in centriole duplication and how this process is spatially and temporally regulated.

Materials and methods

Cell culture and transfection

HEK293T and U2OS cells were grown in DME + 10% fetal bovine serum (Invitrogen). RPE-1 cells were cultured in DME/F12 + 10% fetal bovine serum (Invitrogen). RPE-1 Tet-O::Plk4 GFP-hCent2 cells were provided by B. Tsou (Memorial Sloan-Kettering Center, New York, NY). Plk4 expression was induced by addition of 1 µg/ml doxycycline [Takara Bio Inc.] for 18 h. U2OS cells were arrested in S phase by incubation in 2 µg/ml aphidicolin (Sigma-Aldrich) for 24 h. Plasmids were transfected using Lipofectamine 2000 according to the manufacturer’s instructions (Invitrogen).

Plasmids

Full-length cDNAs of Cep152 [GenBank/EMBL/DDB] accession no. NM_001194998], Plk4 [accession no. NM_014264], and Plx4 [accession no. NM_001089677] were obtained from Thermo Fisher Scientific. The ORFs of Cep152 and Plk4 were amplified by PCR and cloned into pEGFP-C1 [Takara Bio Inc.]. Plk4, Plx4, and Cep152 were cloned into a pEGFP-N1 derivative with a Myc-6×His tag in frame with GFP. Plx4-D154A was made by site-directed mutagenesis (QuickChange II; Agilent Technologies).

Deletion constructs of Cep152 and Plk4 were made by PCR amplification of the indicated regions and ligation into pEGFP-C1 or pCMV-7mch, respectively. Internal deletion constructs for Cep152 and Plk4 were created by triple ligation of two PCR fragments joined by an XmaI site into the same vectors. RNAi-resistant GFP-Cep152 was made by site-directed mutagenesis of pEGFP-C1-Cep152. A total of 13 base pairs were replaced in the two regions of Cep152 hybridizing to the siRNAs without altering the amino acid sequence.

The MBP-Cep152 (1–217) fusion was made by PCR amplification of amino acids 1–217 of Cep152 and ligation into pMal-c2X (New England Biolabs, Inc.). An expression construct of GST fused to Plx4 amino acids 860–942 was made by PCR amplification and ligation into pGEX-AT1 (GE Healthcare) for antibody production. For GFP mRNA transcription, the pGFPPlx4-1 cytomegatovirus promoter was replaced with the CMV promoter from pCMV7-mh. Expression constructs of GFP fused to 6×His and GST used to generate the polyclonal anti-GFP antibody were provided by M. Nachury (Stanford University, Stanford, CA).

Antibodies

Anti-Plx4 antibody was obtained by immunizing rabbits with GST fused to the C-terminal B5 amino acids of Plx4 [Cocalico Biologicals]. Plx4-specific antibodies were purified against GST-Plx4 immobilized on resin (UltraLink Biosupport; Thermo Fisher Scientific) after initially passing the serum over a GST column. The Plx4 antibody recognized human Plx4 specifically as determined by antibody-blocking experiments in U2OS cells and was used at 1.5 µg/ml for immunofluorescence and 0.15 µg/ml for Western blotting. Rabbit anti-Plx4 was obtained from Bethyl Laboratories, Inc. (peptide antigen, residues B25–B75) and diluted 1:1,000 before use. Anti-GFP antibody was obtained by immunizing rabbits [Cocalico Biologicals] with GST-GFP purified from Escherichia coli. The antibody was affinity purified against GFP-6×His bound to Ni-NTA Sepharose beads (Qiagen) and used at 1.5 µg/ml for immunofluorescence and 0.15 µg/ml on Western blots. The polyclonal anti-centrin antibody used for immunofluorescence of centrosomes in Xenopus egg extract was made against Xenopus centrin cloned from a stage 17 cDNA library (NIBI Protein database accession no. AAA79194) and expressed as a His-tagged protein in E. coli. Antibody affinity was purified against the antigen and used at 0.5 µg/ml.

Other antibodies used for immunofluorescence in this study were mouse anti-α-tubulin (GTU-88; Sigma-Aldrich) at 1:1,000, mouse anti-α-actinin (DM1A1; Sigma-Aldrich) at 1:2,000, mouse anti-acetylated α-tubulin (6-11B-1; Abcam) at 1:500, mouse anti-α-tubulin (3e6; Invitrogen) at 1:500, mouse anti–polyglutamateylated tubulin (GT335; provided by C. Janke, Centre de Recherches de Biochimie Macromoléculaire, Montpellier, France) at 1:1,000, mouse anti–centrin-2 (20H5; provided by J. Salisbury, Mayo Clinic, Rochester, MN) at 1:2,000, anti-Sas6 (sc-81431; Santa Cruz Biotechnology, Inc.) at 1:200, and mouse anti-Myo (R610) at 1:500 for immunofluorescence and 1:5,000 on Western blots. Purified rabbit IgG was purchased from Jackson ImmunoResearch Laboratories, Inc.

Xenopus embryos and egg extracts

mRNAs of Plx4 and GFP were transcribed using the mMessage mMachine T7 kit [Applied Biosystems]. Cytosolic factor (CSF) egg extracts were made as described previously [Murray, 1991] and supplemented with 50 mM sucrose. Plx4 mRNA was added to CSF extracts and incubated 1 h before...
Figure 5. Functional interaction of Cep152 and Plk4. (A) Plk4 is lost from the centrosome in cells expressing GFP-Cep152 (1–217). U2OS cells transfected with GFP or GFP-Cep152 (1–217) were fixed 24 h after transfection and labeled with antibodies to GFP, Plk4, and γ-tubulin. Numbered boxes identify enlarged images shown on right. Bars (boxes) 1 µm; (fields) 5 µm. (B) U2OS cells were depleted of Plk4 or Cep152 by siRNA transfection, fixed 72 h after transfection, and labeled with antibodies to Cep152, Plk4, and γ-tubulin. Image pairs represent centrosomes in cells from the same coverslip taken with the same camera settings. (C) U2OS cells transfected for 72 h with either control siRNA (Ctl) or Cep152 siRNAs were arrested in S phase by incubation in aphidicolin for 24 h and fixed and labeled with antibodies to centrin, Sas6, and Cep152. 23% of Cep152-depleted cells had reduced Sas6 labeling on both centrioles (middle), 65% had no Sas6 labeling (bottom), and 10% had centrioles that differed in the amount of Sas6 (n = 150 cells). Exposure and gain conditions were constant for Sas6 image acquisition. (B and C) Bars, 1 µm. (D) Cep152 can be phosphorylated by Plk4 in vitro. Purified GST-Plk4, either wild type (WT) or kinase dead (KD), was incubated in the presence of [32P]ATP with full-length GFP-Cep152 (left) or GFP-Cep152 (1–217; middle) isolated from HEK293T cells by anti-GFP immunoprecipitation (IP) or with MBP-Cep152 (1–217; right) purified from E. coli. Proteins were visualized by Western blotting (Ab) of 0.1× kinase assay input using antibodies to GFP (left and middle) or by Coomassie stain (C) of the gel (right). Bands marked with an asterisk indicate autophosphorylated Plk4. Cep152 32P incorporation was normalized within each gel group. Black lines indicate that gel lanes imaged by different methods were spliced together.
calcium addition to release it into interphase and 2 h after calcium addition at 16°C. Addition of mRNA to mitotic extracts permitted sufficient Plk4 translation to drive de novo structure formation when the extract was released into interphase before the extract reentered mitosis. Centrosome-positive structures formed ~2 h after calcium were added to the CSF extract to release it into interphase; most formed within 30 min of their initial appearance. These structures only form in interphase extract; they do not form in CSF extract incubated with PK4 mRNA even after longer incubation time.

Centrosomes were visualized by incubating extracts on ice for 20 min to depolymerize microtubules and diluting them in 1x BRBB0, 2% Triton X-100, 4% paraformaldehyde, and 0.25% glutaraldehyde for 3 min at room temperature. Samples were centrifuged through a 30% glycerol cushion onto a coverslip at 4°C for 15 min. Coverslips were postfixed in methanol. For aster visualization, an equal volume of CSF extract was added to the interphase extract for an additional 30 min before centrifugation and the ice incubation was omitted. The number of centrioles per field was counted for at least 25 fields per coverslip and averaged to determine the mean centrioles per field. Average centrioles/field was multiplied by fields/coverslip and divided by the μL extract centrifuged onto the coverslip to obtain centrioles/μL extract.

Mass spectrometry
Plk4-D154A with a myc-6xHis C-terminal tag was transcribed and translated in egg extract as described in the Xenopus embryos and egg extracts section. The extract was diluted 10 times in binding buffer (50 mM Tris, pH 7.6, 150 mM NaCl, 0.25% Triton X-100, 1 mM DTT, and protease inhibitors) and incubated with NiNTA agarose beads (Qiagen). Proteins were eluted with reduced glutathione (GSH; Sigma-Aldrich) and were reloaded with anti-Plk4 antibody cross-linked to protein A beads (Affi-Prep; Bio-Rad Laboratories). Proteins were eluted by incubation in sample buffer and run on an SDS-PAGE gel. Silver-stained protein bands present in the Plk4-D154A sample but not in control (at ~125, 170, and 190 kD) were excised and analyzed by mass spectrometry at the Stanford Vincent Coates Foundation Mass Spectrometry Laboratory (http://mass-spect.stanford.edu). Bands were digested with trypsin (Promega). A LCQ Deca XP Plus mass spectrometer was used (Thermo Fisher Scientific). The database was composed of Xenopus and Xenopus tropicalis protein sequences archived in the National Center for Biotechnology Information (NCBI). Proteins were identified on the basis of at least two peptide matches with a probability >95%. IOD548794 was identified as Wdr33 in Homologene (NCBI).

Immunoprecipitations
HEK293T cells were transiently transfected, incubated for 24 h, washed with PBS, and lysed in NP-40 buffer. Insoluble material was pelleted, and soluble material was incubated with rabbit anti-GFP antibodies and then with protein A beads (Affi-Prep). Beads were washed in NP-40 buffer, eluted in sample buffer, and run on SDS-PAGE gels.

Immunofluorescence
Cells were grown on coverslips and fixed in methanol for indirect immunofluorescence. After rehydration in PBS, cells were blocked in 3% BSA (Sigma-Aldrich) in PBS + 0.1% Triton X-100. Coverslips were incubated in primary antibodies diluted in blocking solution as previously indicated (see Antibodies), and Alexa Fluor 488-, 594-, or 350-conjugated secondary antibodies were diluted 1:200 in blocking solution (Invitrogen). Double labeling with monoclonal mouse antibodies was performed using secondary antibodies that recognize specific isotypes of mouse IgG (Invitrogen). For double labeling with rabbit anti-Plk4 and rabbit anti-Cep152, a monovalent goat anti-rabbit Fab fragment (Jackson ImmunoResearch Laboratories, lot 005238) was used as a blocking agent at 100 μg/mL. Coverslips of cells or extract samples were imaged using Openlab software (version 4.0.4; Perkin Elmer) on a microscope (Axiovert 200M; Carl Zeiss, Inc.) with Plan Neofluar 100X 1.3 NA objectives. Images were captured using a cooled charge-coupled device camera (Orca ER; Hamamatsu Photonic) and were processed using Photoshop [Adobe].

For colocalizing Cep152, Saxd, centrin, and γ-tubulin, a z stack of 11–13 optical sections at 0.3-μm intervals was obtained using an epi-fluorescence inverted microscope (IX70; Olympus) in an imaging station (DeltaVision; Applied Precision) maintained by the Beckman Center Cell Sciences Imaging Facility with a 100X 1.35 NA objective (Olympus). Images were acquired with a charge-coupled device camera (CH5300; Photometrics). Images were deconvolved using the constrained iterative algorithm and point-spread functions supplied with the DeltaVision system. Deconvoluted stacks were merged into a single image using maximum intensity projections.

RNAi
Plk4 was depleted using an siRNA with the sequence 5'-CGTGGTAGAC- TAGTCACCCGA-3' as previously described (Habedanck et al., 2005). Cep152 was depleted using a pool of two siRNA duplexes: 5'-GCGGATG- CAACTGGAATCTA-3', as previously described (Kleylein-Sohn et al., 2007), and 5'-GCATGGAGTGAGGACCTA-3'. All siRNAs were synthesized by Thermo Fisher Scientific. siRNAs were transfected into U2OS and RPE-1 cells using Lipofectamine RNAiMax following the manufacturer's instructions (Invitrogen). For Cep152 depletion of 72 to 96 h, siRNAs were serially transfected every 24 h two and three times, respectively. An siRNA against luciferase was used as a control (Thermo Fisher Scientific). Western blot bands were quantified using an imager (Typhoon9210; GE Healthcare) and ImageQuant software (version 2003.01; GE Healthcare).

Kinase assay
Purified GST-Plk4 and GST-Plk4-D154A were made and kinase assays were performed as previously described (Holland et al., 2010). GFP, GFP-Cep152, and GFP-Cep152 (1–217) were immunoprecipitated from HEK293T cells as described above and added to the kinase reaction on the beads. MBP and MBP-Cep152 (1–217) were purified from E. coli using amylose resin (New England Biolabs, Inc.). Kinase reactions were stopped in sample buffer and run on SDS-PAGE gels. Dried gels were exposed to a storage phosphor screen (GE Healthcare), 32P incorporation was imaged using a Typhoon9210, and bands were quantified with ImageQuant. Samples for Western blots were eluted off the beads with sample buffer and run on SDS-PAGE gels.

Online supplemental material
Fig. S1 shows that Plk4-induced de novo objects have the same properties as centrioles and centrosomes. Fig. S2 shows that Plk4 and Cep152 interact and the conservation of Cep152. Fig. S3 shows rescue of the Cep152 depletion phenotype by RNAi-resistant Cep152. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201006049/DC1.

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