MISP is a novel Plk1 substrate required for proper spindle orientation and mitotic progression

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Precise positioning of the mitotic spindle determines the correct cell division axis and is crucial for organism development. Spindle positioning is mediated through a cortical machinery by capturing astral microtubules, thereby generating pushing/pulling forces at the cell cortex. However, the molecular link between these two structures remains elusive. Here we describe a previously uncharacterized protein, MISP (C19orf21), as a substrate of Plk1 that is required for correct mitotic spindle positioning. MISP is an actin-associated protein throughout the cell cycle. MISP depletion led to an impaired metaphase-to-anaphase transition, which depended on phosphorylation by Plk1. Loss of MISP induced mitotic defects including spindle misorientation accompanied by shortened astral microtubules. Furthermore, we find that MISP formed a complex with and regulated the cortical distribution of the +TIP binding protein p150glued, a subunit of the dynein–dynactin complex. We propose that Plk1 phosphorylates MISP, thus stabilizing cortical and astral microtubule attachments required for proper mitotic spindle positioning.

Introduction

The determination of the correct cell division axis is crucial for organism development and is mediated by the precise positioning of the mitotic spindle (Ahringer, 2003; Gönczy, 2008). External positioning signals are transmitted into the cell via the cell cortex (Théry et al., 2005; Toyoshima and Nishida, 2007) and relayed to the mitotic spindle through pulling forces acting on astral microtubules (MTs) attached to cortical structures (Grill et al., 2003). These cortical cues are spatially defined by retraction fibers modulating the positioning of actin regulators and therefore force generation (Théry et al., 2005; Fink et al., 2011). Moreover, astral MTs are engaged with these cortical structures through so-called +TIPs including adenomatous polyposis coli (APC), CLASPs, and the dynein–dynactin complex (Grill et al., 2003). These cortical cues are spatially defined by retraction fibers modulating the positioning of actin regulators and therefore force generation (Théry et al., 2005; Fink et al., 2011). Moreover, astral MTs are engaged with these cortical structures through so-called +TIPs including adenomatous polyposis coli (APC), CLASPs, and the dynein–dynactin complex, which have been shown to regulate spindle orientation and positioning (O’Connell and Wang, 2000; Schuyler and Pellman, 2001; Rogers et al., 2002; Mimori-Kiyosue and Tsukita, 2003; Samora et al., 2011). The cortically localized dynein–dynactin complex is believed to provide pulling forces on astral MTs and is recruited by heterotrimeric G proteins/LGN/NuMA during spindle positioning in Caenorhabditis elegans embryos, Drosophila, and human cells (Du and Macara, 2004; Bowman et al., 2006; Siller et al., 2006; Couwenbergs et al., 2007; Nguyen-Ngoc et al., 2007; Woodard et al., 2010). However, the detailed mechanisms underlying the correct positioning and orientation of the mitotic spindle are still not fully understood and further investigation is needed to identify potential missing components involved in this process.

Polo-like kinase 1 (Plk1) is a major mitotic kinase important for many processes such as centrosome maturation, kinetochore–microtubule attachment, and furrow ingression (Petronczki et al., 2008). Plk1 binds to its interaction partners mainly through its Polo box domain (PBD), which is a phosphopeptide recognition motif that recognizes prephosphorylated, “primed” proteins (Elia et al., 2003a,b). This binding mechanism dynamically targets Plk1 to different subcellular localizations in mitosis like the centrosomes, kinetochores, spindle microtubules, and the central spindle (Petronczki et al., 2008; Elia et al., 2003a,b). The Polo box domain of Plk1 is mainly involved in the recruitment of substrates and it has been shown that Plk1 phosphorylates MISP, thus stabilizing cortical and astral microtubule attachments required for proper mitotic spindle positioning.
Johmura et al., 2011). Recently, it was shown that Plk1 phosphorylates Dvl2, a component of the Wnt signaling pathway, to regulate mitotic spindle orientation (Kikuchi et al., 2010). Moreover, spindle pole–localized Plk1 controls spindle positioning and orientation by regulating the binding between dynein–dynactin and its upstream cortical targeting factors NuMA and LGN (Kiyomitsu and Cheeseman, 2012).

Here, we report the identification of MISP, a previously uncharacterized actin-binding protein. We show that MISP is a mitotic phosphoprotein and substrate of Plk1. Phosphorylation of MISP by Plk1 is required for progression through mitosis and correct spindle orientation. Depletion of MISP impairs mitosis at the metaphase-to-anaphase transition with fragmented centrosomes, scattered chromosomes, and misoriented, rocking spindles. Furthermore, interference with MISP function causes a severe diminution of astral MTs. We find that MISP also associates with and negatively regulates the cortical distribution of p150

**Results**

**Identification of MISP as a novel mitotic phosphoprotein and Plk1 substrate**

To identify novel substrates and effectors of Plk1 during mitosis, we immunoprecipitated endogenous Plk1 from HeLa cells that were synchronized in late mitosis by nocodazole block and 1.5 h release, and subjected the immunoprecipitate to an in vitro kinase assay. Phosphorylated bands were analyzed by mass spectrometry resulting in the identification of C19orf21 (Fig. S1, A and B). Hereafter, we refer to C19orf21 as MISP (mitotic interactor and substrate of Plk1). MISP is a previously uncharacterized protein conserved among vertebrates and *Drosophila* with homologies to proteins of the AKAP family (Fig. S1 C). To analyze the function of MISP, rabbit polyclonal antibodies were raised against the full-length protein. In Western blots, the antibody recognized a major band at the expected molecular weight of ~75 kD and a slower migrating band, which were largely reduced in MISP-depleted cells using two different siRNAs (OI1 and OI2; Fig. S1 D). To investigate whether MISP protein levels are regulated during the cell cycle, HeLa cells were synchronized with a double thymidine block at the G1/S boundary and released for different time points. Cell cycle progression was controlled by Western blot analysis of cell cycle marker proteins and monitored by FACS analysis. As shown in Fig. 1 A, MISP was only weakly expressed in G1 and S phases of synchronized HeLa cells, with increasing protein levels and slower migrating bands appearing stepwise in G2/M phases and persisting until the end of mitosis. The slower migrating form of MISP disappeared in response to λ-phosphatase treatment, indicating that MISP is a phosphoprotein (Fig. 1 B).

To assess the contribution of Plk1 function to the regulation of MISP during mitosis, we first sought to confirm the interaction between MISP and Plk1. As seen in Fig. 1 C, endogenous MISP was present in Plk1 immunoprecipitates. In addition, interactions between ectopically expressed Flag-MISP and Plk1 could also be detected in vivo (Fig. 1 D). Interestingly, Plk1 was found to bind to the highest phosphorylated form of MISP (Fig. 1 C). Plk1 is known to bind to substrates in a phospho-specific manner via its PBD (Elia et al., 2003a,b). To test this, we made use of a far Western blot assay (Neef et al., 2003). The GST-PBD of Plk1 was able to bind to the highest phosphorylated form of precipitated Flag-MISP in mitosis, while this interaction was weakened in asynchronous cells and abolished by λ-phosphatase treatment (Fig. 1 E, left). Moreover, a PBD mutant (W414F/H538A/K540M, FAM) deficient in phospho-peptide binding (Elia et al., 2003a,b) attenuated the ability to interact with Flag-MISP (Fig. 1 E, right; and Fig. S1 E). Then, the interaction of MISP with Plk1 was mapped in a GST pull-down assay. Different C-terminal truncations of Flag-MISP were generated and incubated with GST-Plk1 or Plk1 PBD. We found that the last 179 amino acids (aa 501–679) of the C-terminal MISP domain are required for binding to Plk1 and Plk1 PBD (Fig. S1, F and G; and unpublished data). Interestingly, the C-terminal domain is the most conserved part in MISP among different orthologues (Fig. S1 C).

To verify whether MISP is a substrate of Plk1, in vitro kinase assays with recombinant GST-MISP and His-Plk1 were performed in the presence or absence of the Plk1 inhibitor BI2536. As shown in Fig. 2 A, a strong phosphorylation of GST-MISP by Plk1 was detected and was weakened in the presence of BI2536. Furthermore, interference with Plk1 activity in mitosis by siRNA-mediated depletion led to a pronounced reduction of MISP phosphorylations (Fig. 2 B). Taken together, we identified MISP as a novel substrate and interaction partner of Plk1 in mitosis.

Cdk1 is an important mitotic kinase and was shown to be one of the major priming kinases for Plk1 binding. Thus, we attempted to detect an interaction between MISP and Cdk1. Endogenous Cdk1–cyclin B complexes interacted with endogenous MISP in mitotic HeLa cells (Fig. S2 A). Interestingly, Cdk1 interacted preferentially with a weakly phosphorylated, faster migrating form of MISP, hinting at a potential role of Cdk1 as the priming kinase for Plk1 phosphorylation. As expected, Cdk1 strongly phosphorylated GST-MISP in vitro and the phosphorylation was abolished in the presence of the Cdk inhibitor roscovitine (Fig. S2 B). The strong phosphorylation by Cdk1 led to an upshift of GST-MISP in the Coomassie gel reminiscent of mitotic MISP (unpublished data).

We next determined the potential phosphorylation sites of both kinases on MISP by mass spectrometry analysis after in vitro phosphorylation. Six potential sites for Plk1 and nine potential sites for Cdk1 were identified on MISP (Fig. S2 C). Mutations of the respective S/T residues to nonphosphorylatable alanines (9AC for Cdk1 and 7AP for Plk1) reduced the phosphorylation of GST-MISP by Plk1 (Fig. 2 C) and significantly abolished Cdk1 phosphorylation (Fig. 2 D). Moreover, ectopic expression of GFP-tagged versions of MISP revealed an accelerated electrophoretic mobility for both mutants in nocodazole-arrested HeLa cells (Fig. S2 D). Importantly, the PBD of Plk1 was unable to bind to the precipitated Flag-MISP 9AC mutant while it still bound to Flag-MISP WT and 7AP mutant (Fig. 2 E). Thus, Plk1 binds to MISP in mitosis through priming phosphorylation by Cdk1.
MISP is an actin-binding protein that regulates the metaphase-to-anaphase transition

Next, we determined the subcellular localization of MISP during the cell cycle. MISP was found to be predominantly localized to cortical actin structures during interphase and mitosis as shown by immunofluorescence staining with MISP antibodies and phalloidin (Fig. 3 A). We also observed a partial colocalization of MISP with cytoplasmic actin filaments. A clear colocalization with actin filaments was observed upon overexpression of MISP in HeLa (Fig. S2 E) or RPE-1 cells (not depicted). MISP WT and the phospho-mutants (6DP, 7AP, 9AC) showed

![Figure 1](image_url)
a similar cortical localization in mitotic cells (Fig. S2 F). We were not able to detect an obvious colocalization of MISP with the mitotic spindle (Fig. S3 A) or with Plk1 (Fig. S3 B), although it was found in the mitotic spindle phosphoproteome of Plk1 (Santamaria et al., 2011). We propose that the cytoplasmic part of MISP associates with Plk1 in vivo. MISP’s cortical localization was disrupted by depolymerization of the actin network in response to cytochalasin D (Cyto. D) treatment (Figs. 3 B and S2 E) or siRNA-mediated depletion (Fig. S3 C), pointing to a specific localization of MISP at the cell cortex. We then set out to identify the potential of MISP to bind to actin filaments using an actin cosedimentation assay. We were able to detect substantial cosedimentation of GST-MISP with polymerized actin (F-actin; Fig. 3 C). Thus, MISP is an actin-associated protein.

To study the role of MISP during the cell cycle, we depleted endogenous MISP with Ol1 or Ol2. MISP depletion led to a marked increase in the number of mitotically rounded up cells (unpublished data). To confirm this observation, we made use of the mitotic marker p-Ser10 Histone H3 (p-Ser10 H3). We observed a three- to fivefold increase in the mitotic index evaluated by p-Ser10 H3 staining (Fig. 4, A and B) after MISP siRNA
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cytokinesis during the monitored period with an average time of 247 min from nuclear envelope breakdown (NEB) to anaphase transition (Videos 1 and 2) compared with 42 min (Video 3) in control cells (Fig. 4, C and D); the remainder ended with scattered chromosomes or cell death. After NEB, both control- and MISP-depleted cells exhibited bipolar spindle assembly; over 80% of MISP-depleted cells properly aligned their chromosomes during early mitosis, although some cells showed a slight delay compared with the control (Fig. 4, C and E; Videos 1–5).

While control cells progressed to anaphase immediately after metaphase chromosome alignment, MISP-depleted cells were arrested with rocking and flipping spindles in metaphase in which the entire spindle oscillated within the cell for several treatment. To ensure that this phenotype is not due to off-target effects, we performed a rescue experiment by generating an siRNA (O12)-resistant version of GFP-MISP that was coexpressed during siRNA-mediated depletion of endogenous MISP. GFP-MISP was able to reduce the mitotic index nearly back to control levels, which was not the case upon expression of the GFP control (Fig. S3, D and E). We next used time-lapse video microscopy to confirm the effect of MISP ablation and gain further insight into mitotic progression in real time. MISP was depleted in HeLa cells stably expressing GFP-α-tubulin/RFP-H2B or GFP-H2B and cells were monitored for 8–12 h after 24 h or 28 h of siRNA transfection. After MISP knockdown, around half of the mitotic cells entered anaphase and completed cytokinesis during the monitored period with an average time of 247 min from nuclear envelope breakdown (NEB) to anaphase transition (Videos 1 and 2) compared with 42 min (Video 3) in control cells (Fig. 4, C and D); the remainder ended with scattered chromosomes or cell death. After NEB, both control- and MISP-depleted cells exhibited bipolar spindle assembly; over 80% of MISP-depleted cells properly aligned their chromosomes during early mitosis, although some cells showed a slight delay compared with the control (Fig. 4, C and E; Videos 1–5). While control cells progressed to anaphase immediately after metaphase chromosome alignment, MISP-depleted cells were arrested with rocking and flipping spindles in metaphase in which the entire spindle oscillated within the cell for several

Figure 3. MISP is an actin-associated protein. (A) Localization of MISP (gray; red in merge) in interphase and different indicated mitotic stages of HeLa cells was shown by costaining with MISP antibody, Atto 488 phalloidin (green), and Hoechst 33342 (blue). Bars, 10 µm. Insets showed higher magnification of the framed regions. Bars, 5 µm. (B) HeLa cells were incubated with 2 µM of the actin-depolymerizing agent cytochalasin D (Cyto. D) for 10 min and analyzed by immunofluorescence. MISP, gray; red in merge; Atto 488 phalloidin, green. Bars, 10 µm. Insets showed higher magnification of the framed regions. Bars, 5 µm. (C) Actin cosedimentation assay with polymerized nonmuscle β-actin and GST-MISP or GST control. P, pellet; S, supernatant.
Figure 4. Depletion of MISP impairs the metaphase-to-anaphase transition. (A) Asynchronous HeLa cells were stained for p-Ser10 H3 (red) and α-tubulin (green) 48 h after control or MISP Ol2 siRNA treatment. Bar, 50 µm. (B) Quantification of the mitotic index in HeLa cells transfected with control or MISP siRNA (Ol1/Ol2) by evaluating p-Ser10 H3 staining. Results represent the mean of three different experiments (n = 200 cells per experiment). (C) HeLa cells stably expressing GFP-α-tubulin/RFP-H2B were transfected with control or MISP Ol2 for 24 h followed by live-cell imaging for 8 h. Frame series of movies of control and MISP knockdown cells with continuous time points (minute) are shown. Bar, 10 µm. (D) Box plot of C displaying the time from NEB to anaphase;
hours. The cortical integrity of most MISP-depleted cells remained intact, as visualized by phase-contrast imaging shown in Video 4 and by actin staining in fixed cells (Fig. S3 C).

The spindle assembly checkpoint (SAC) is a control mechanism that arrests cells with spindle defects or defective kinetochore–microtubule attachments at the metaphase-to-anaphase transition (Musacchio and Salmon, 2007). To investigate the activation state of the SAC after MISP depletion, we performed immunofluorescence analysis of fixed HeLa cells with an antibody against BubR1, a well-known marker of an active SAC. We detected clear BubR1 staining on kinetochores of cells with misaligned chromosomes, and observed a weak localization of BubR1 on kinetochores of cells with aligned chromosomes (Fig. S3 F, middle and bottom panels, respectively). We also detected increased phosphorylation of BubR1 after MISP depletion by Western blot (Fig. 4 F), indicating an active SAC (Mao et al., 2003). The remaining BubR1 signal upon MISP depletion prevents SAC satisfaction, thereby leading to a delay in the metaphase-to-anaphase transition. Furthermore, co-depletion of another core SAC protein, Mad2, together with MISP completely abolished the arrest in mitosis (Fig. 4 F; and Fig. S3, G and H). Thus, the arrest in metaphase as observed in response to MISP depletion is mediated by the SAC.

As MISP is highly phosphorylated during mitosis by Plk1 and Cdk1, we asked whether phosphorylation contributes to the function of MISP in regulating mitotic progression. We depleted endogenous MISP from HeLa cells and coexpressed GFP-tagged siRNA-resistant versions of MISP including WT, 7AP, 9AC, and 6DP (phospho-mimicking aspartic acid mutant of MISP for Plk1; Fig. S3 I). We found that both nonphosphorylatable mutants of MISP (7AP and 9AC) for Plk1 and Cdk1 were not able to rescue the observed mitotic arrest, whereas expression of the WT and the 6DP mutants successfully reduced the mitotic index nearly back to control levels (Fig. 4 G). From these data, we conclude that phosphorylation of MISP by Plk1 is required for its function in regulating the metaphase-to-anaphase transition.

**Loss of MISP leads to spindle misorientation**

As MISP knockdown delays anaphase entry, we set out to further analyze the effects of MISP depletion in mitotic cells in more detail. Close inspection of the mitotic spindle stained for α-tubulin and the centromeral marker γ-tubulin in MISP-depleted cells revealed a marked increase in several mitotic defects such as chromosome misalignment, spindle tilt, disrupted spindle poles, and movement of the spindle out of the cell center to the cortex, with the majority of the cells showing one or a combination of the described defects (Fig. 5, A and B). The disruption of the spindle poles was further confirmed by inspection of another centrosomal marker, pericentrin (Fig. S4 A), and the centriolar marker Cep135 (Fig. S4 B). The observed multiple centrosome dots were not caused by centrosome overduplication, as just four centrioles were detected in MISP-depleted mitotic cells based on Cep135 staining. As we did not observe obvious disrupted spindle poles (Videos 1 and 2) and chromosome congression defects (Videos 1, 2, and 4) during early mitosis in MISP-depleted cells by live-cell imaging, we speculate that centrosome splitting and chromosome misalignment after MISP knockdown may be caused by a long-term metaphase arrest-induced “cohesion fatigue”, which leads to sister chromatid separation followed by centriole disengagement and chromosome scattering (Daum et al., 2011; Stevens et al., 2011). To test this, we silenced the active SAC by co-depletion of Mad2 together with MISP. The percentage of metaphase cells with disrupted poles after Mad2 co-depletion was dramatically decreased compared with MISP knockdown alone (Fig. S4 C), pointing to an indirect role of MISP in centrosome splitting. The majority of MISP-depleted cells properly aligned chromosomes during early mitosis (Fig. 4 E). We propose that the chromosome misalignment, however, occurred afterward as shown in Video 4, and seemed also to be a secondary effect of prolonged metaphase arrest caused by uncoordinated cohesion loss. The SAC is then efficiently re-activated to maintain the scattered state of chromosomes (Stevens et al., 2011). Additionally, the split spindle poles in MISP knockdown cells had similar spindle microtubule nucleation activity as in control cells, validated by a microtubule regrowth assay (Fig. S4 D).

To characterize the observed spindle tilt more closely, the orientation of the mitotic spindle was determined in MISP-depleted HeLa cells grown on fibronectin-coated coverslips in relation to the substratum by confocal microscopy of the spindle and the spindle poles (Fig. 5 C). Inspection of the spindle poles revealed that in MISP-depleted cells, 29 ± 7% of the cells showed a spindle angle α of more than 20° as compared with 5 ± 7% in control cells (Fig. 5 D). The average spindle rotation increased to 14.2° in MISP-depleted cells from 8.6° in control cells (Fig. S4 E). To rule out the possibility that spindle misorientation in MISP-depleted cells is a consequence of an extended metaphase block, we arrested cells in metaphase in the presence of MG132 and analyzed spindle angles in control and MISP siRNA-treated cells as described in Fig. 5, C and D. Similar as observed before by live-cell imaging (Toyoshima et al., 2007; Chan et al., 2009), a short-time prolonged metaphase arrest by MG132 treatment did not markedly rotate the spindle along z-axis and attenuate the observed effect on spindle orientation after MISP knockdown (Fig. S4 F). Furthermore, down-regulation of CENP-E required for chromosome alignment leads to delayed metaphase-to-anaphase transition but without having an obvious effect on spindle orientation (Figs. 5 E

\[ n = 20 \text{ cells each from three independent experiments. (E) Percentage of mitotic cells with aligned chromosomes during early mitosis in control and MISP siRNA-treated cells evaluated by live-cell imaging (} n = 100 \text{ cells from two independent experiments). (F) HeLa cells were treated with control or MISP siRNAs with and without additional depletion of Mad2 for 48 h. Samples were analyzed by Western blot. Arrowhead indicates phosphorylated BubR1 with retarded electrophoretic mobility. (G) HeLa cells transiently transfected with GFP alone or siRNA-resistant versions of GFP-MISP WT/7AP/9AC/6DP were treated with MISP or control siRNA for 48 h. The mitotic index was evaluated (} n = 200 \text{ cells) by p-Ser10 H3 staining, respectively from three independent experiments. Error bars in D, E, and G represent SD. Student's t-test was used to calculate p-value for comparison of control- and MISP-depleted samples.} \]
Figure 5. Loss of MISP leads to spindle misorientation. (A) HeLa cells treated with MISP or control siRNA for 48 h were stained for α-tubulin (green), γ-tubulin (red), and with Hoechst 33342 (blue). (B) Quantification of the described mitotic defects in control or MISP siRNA-treated cells. n = 50 cells each from three independent experiments. (C) Representative images of z-sections (0.4 µm per stack) with maximum spindle pole intensity and a side view of a 3D-reconstruction of the depicted cell with the spindle angle α. Cells were stained for α-tubulin (red), γ-tubulin (green), and Hoechst 33342 (blue). (D) Quantification of the percentage of metaphase cells with indicated spindle angles as described in C; n = 150 cells from three independent experiments. (E) HeLa cells treated with CENP-E, MISP, or control siRNA for 48 h were stained for α-tubulin (green), γ-tubulin (red), and with Hoechst 33342 (blue).
and S4 G; Chan et al., 2009; Dunsch et al., 2012), indicating a specific role of MISP in regulating this process. Moreover, spindle misorientation caused by MISP depletion can be rescued upon expression of MISP WT and the Plk1 phospho-mimetic MISP 6DP mutant but not the nonphosphorylatable MISP 7AP and 9AC mutants (Fig. 5 F). These results suggest that Plk1 phosphorylation positively regulates the function of MISP in spindle orientation.

Next, we set to analyze the consequence of the observed mitotic defects on cell survival. The detected severe malfunctions in mitotic progression led to a marked increase in the cleavage of the apoptotic marker PARP (Fig. S4 H), as well as a fivefold increase in AnnexinV-FITC–positive cells (Fig. S4 I). Therefore, the mitotic arrest caused by MISP ablation and the associated mitotic defects likely ultimately led to cell death.

### MISP stabilizes astral microtubules

We then analyzed the mechanism of spindle misorientation induced by MISP depletion in more detail. Interactions of astral MTs with cortical structures are necessary for proper spindle orientation (Toyoshima and Nishida, 2007; Toyoshima et al., 2007). Quantification of the relative astral MTs signal intensity in fixed α-tubulin–stained cells demonstrated that MISP knockdown led to over 60% reduction in relative astral MT intensity compared with control cells (Fig. 6, A and B). The fluorescence intensity of the spindle MTs was comparable in both siRNA-treated cells (Fig. S5 A). Moreover, the spindle MTs in metaphase cells with aligned chromosomes were cold-stable after MISP depletion (Fig. S5 B) and also exhibited similar regrowth activity as control cells (Fig. S4 D), indicating that MISP depletion does not obviously alter the overall spindle microtubule stability and dynamics. To visualize the microtubule plus ends, we stained for the microtubule +TIP binding protein EB1 that was present at the tips of spindle MTs, but only 34% of the cells showed EB1 signal near the cortex (Fig. 6, C, bottom inset). No difference in overall EB1 protein levels was observed (unpublished data). The reduced astral MTs were also observed in live-cell imaging tracked by EB3 (Videos 6 and 7). We propose that MISP knockdown disturbs the attachment of astral MTs to the cortex and thereby leading to their destabilization and shortening and thus spindle mispositioning. It was previously shown that astral MT interaction with the cell cortex participates in spindle orientation (Théry et al., 2005; Toyoshima and Nishida, 2007). Indeed, MISP can be detected in the vicinity of astral MTs at the cell cortex in fixed cells (Fig. 6 D).

Given that MISP is an actin-binding protein (Fig. 3) and was not found to localize at the mitotic spindle (Fig. S3 A), the interaction between MISP and astral MTs is likely to be indirect. This was further confirmed by using an in vitro MT-binding assay. Unlike the MT-associated protein (MAPF), MISP was unable to efficiently copellet with taxol-polymerized MTs in vitro (Fig. S5 C). We speculate that MISP indirectly regulates astral MT stability probably by disassociating them from the cell cortex and microtubule stabilizers. The loss of astral MTs in MISP-depleted cells can be rescued after expression of MISP WT or 6DP mutant but not 7AP or 9AC mutants (Fig. 6 E), suggesting that Plk1 phosphorylation on MISP is required for stabilizing astral MTs and their cortical attachment.

### MISP interacts with and regulates the cortical distribution of p150\textsuperscript{glued}

The cortically localized dynein–dynactin complex is known to play a crucial role in spindle orientation and positioning during embryonic and somatic cell divisions (Couvernbergs et al., 2007; Nguyen-Ngoc et al., 2007; Toyoshima et al., 2007; Kotak et al., 2012). To identify MISP-interacting proteins in regulation of spindle orientation, we performed a pull-down assay with GST-MISP using HeLa mitotic cell lysates followed by mass spectrometry analysis. In addition to actin, we also identified p150\textsuperscript{glued}, a subunit of the dynactin complex and a +TIP binding protein (Fig. S5 D). We first confirmed the interaction by endogenous p150\textsuperscript{glued} immunoprecipitation (Fig. 7 A). Next, we sought to investigate whether MISP would form a complex with microtubules and p150\textsuperscript{glued} in vivo. MTs from mitotic HeLa cells were polymerized or depolymerized by taxol or nocodazole treatment, respectively, and then pelleted by high-speed centrifugation. MISP appeared to cosedent with taxol-stabilized MTs together with p150\textsuperscript{glued} and actin, whereas MEK1/2 remained in the supernatant excluding cross-contamination with cytoplasmic proteins (Fig. 7 B). Previous findings also suggest that MISP associates with the mitotic spindle, as it was identified in mitotic spindle phosphoproteome (Nousiainen et al., 2006; Santamaria et al., 2011). However, depletion of either p150\textsuperscript{glued} or MISP did not reduce cosedimentation of the other with taxol-stabilized MTs from mitotic cells (Fig. S5 E), suggesting that cytoplasmic MISP might associate with spindle microtubules in vivo via other microtubule-binding protein(s). To examine the effects of MISP depletion on p150\textsuperscript{glued} localization, we treated HeLa cells with MISP or control siRNA and processed them for confocal immunofluorescence analysis after preextraction of the cytoplasm. p150\textsuperscript{glued} was found to localize at the spindle and astral microtubules, and cell cortex in control siRNA-treated cells (Fig. 7 C, top); whereas after MISP depletion, in addition to loss of the localization at astral MTs, ~51% of the metaphase cells also showed accumulated and distributed...
Figure 6. **MISP stabilizes astral MTs.** (A) MISP or control siRNA-treated HeLa cells were stained with α-tubulin (green) and Hoechst 33342 (blue). (B) Schematic overview and quantification of the observed metaphase spindles. The image is the control cell shown in A. The intensities of the spindle (I_{spindle}) and the total cell (I_{total}) were determined with ImageJ software. Relative astral MT intensity (I_{astral, rel}) was calculated by \( \frac{I_{total} - I_{spindle}}{I_{spindle}} \) and control value was set to 1. \( n = 20 \) cells each from three independent experiments. (C) RFP-tubulin HeLa stable cells were transfected with MISP or control siRNA and stained for EB1 (green) and Hoechst 33342 (blue). Enlargements of the insets show EB1 localization in the cortical region (dashed lines). Right panel shows quantification of the percentage of metaphase cells with EB1-MT at the cortex; \( n = 50 \) cells each from three independent experiments. (D) Co-staining of MISP (red), α-tubulin (green), and Hoechst 33342 (blue) in fixed HeLa cells. Inset shows higher magnification of the framed region. (E) HeLa cells transfected with control or MISP siRNA, or MISP siRNA under expression of GFP or siRNA-resistant versions of GFP-MISP WT/6DP/7AP/9AC were stained for α-tubulin. Top, representative images with z-projection; bottom, quantification of percentage of metaphase cells with astral MTs. Bars: (A, C-E) 10 μm; (C and D, insets) 2.5 μm. Error bars represent SD. Student’s t-test was used to calculate p-value for comparison of control and experimental samples.
cortical localization of p150\textsubscript{fluid} compared with \(\sim\)11% in the control siRNA-treated cells (Fig. 7, C and D). Next we investigated the impact of Plk1 phosphorylation on MISP in regulating p150\textsubscript{fluid} cortical recruitment. As shown in Fig. 7, E and F, the distributed p150\textsubscript{fluid} at the cortex could be partially rescued by expression of MISP WT and 6DP mutants but not 7AP or 9AC mutants, although there was no obvious difference in the binding of MISP WT and phospho-mutants to p150\textsubscript{fluid} (Fig. S5 F). Therefore, the regulation of the cortical distributed dynactin turnover by MISP is probably controlled through its effects on astral MTs. Taken together, these results indicate that MISP interacts with and regulates the distribution of dynactin at the cell cortex in a Plk1-dependent manner.

Discussion

Our data concur to identify the novel actin-binding protein MISP (C19orf21) as an important regulator of mitotic spindle orientation by stabilizing astral MT attachment to the cell cortex. As MISP is a so far uncharacterized protein, we analyzed the MISP sequence within different species including human, Xenopus, and Drosophila (Fig. S1 C). We identified uncharacterized sequence motifs, a conserved N-terminal region, a small repeated motif, and a conserved C-terminal region, which are also present in AKAP2 (AKAP-KL), a protein that is associated with the cortical actin cytoskeleton (Dong et al., 1998). Proteins of the AKAP family like pericentrin or AKAP450 are involved in microtubule nucleation and spindle pole integrity (Doxsey et al., 1994; Keryer et al., 2003). Moreover, moesin, the sole Drosophila member of the ERM (ezrin/radixin/moesin) family of proteins that includes the AKAP protein ezrin was shown to be required for cortical stability and spindle positioning (Carreno et al., 2008; Kunda et al., 2008). It is therefore tempting to speculate whether MISP is a new member of the AKAP family orchestrating actin cytoskeleton communication with astral MTs and regulating spindle positioning.

Once formed, the mitotic spindle must be properly positioned to establish the correct cell division plane. Insufficient attachment of the astral MT plus ends to the cell cortex causes spindle misorientation (Théry et al., 2005; Toyoshima and Nishida, 2007). However, the mechanisms controlling astral microtubule interactions at the cortex are poorly understood. Here we propose that MISP, a novel actin-binding protein, is involved in stabilizing astral MT attachment to the cortex during mitosis. Ablation of MISP function causes multiple mitotic defects, in particular spindle misorientation and a SAC-dependent delay in the metaphase-to-anaphase transition. Previous findings indicate that spindle misorientation could lead to a delayed anaphase onset in human cells, but most cells with misoriented spindles are eventually able to undergo anaphase (Delaval et al., 2011; Samora et al., 2011; Dunsch et al., 2012). How misoriented spindles prevent SAC satisfaction and cause mitotic delay still remains elusive. Although the majority of MISP-depleted cells properly aligned their chromosomes during early mitosis (Fig. 4 E), chromosome congression defects were observed afterward in both fixed cells and live-cell imaging caused by prolonged metaphase arrest (Fig. 5 B and Video 4). As shown in Fig. 3 F, the BubR1 signal was clearly detected in cells with misaligned chromosomes (middle panel), and also weakly observed in cells with aligned chromosomes (bottom panel) indicative of incomplete removal of SAC proteins. The slight BubR1 staining on the aligned chromosomes is probably due to insufficient kinetochore–microtubule attachments or lack of tension in the intra-kinetochore region between sister chromatids, thus delaying anaphase onset. It remains possible that MISP plays additional roles in spindle checkpoint regulation through other binding partners. Based on our live-cell imaging data, around half of MISP-depleted mitotic cells completed cell division during the monitored period, while the left mitotic cells with misoriented spindles ended with chromosome scattering or cell death, indicating roles of MISP in both spindle orientation and anaphase onset regulation. In this study, we characterized the function of MISP in the regulation of spindle orientation.

The most notable spindle orientation defect in the absence of MISP was a significant loss or shortening of astral MTs. This phenotype observed could be either caused by a failure of astral MT attachment to the cell cortex or by a defect in microtubule nucleation. However, a direct role of MISP in regulating the assembly of astral MT arrays is unlikely because we did not observe an obvious change in the levels of microtubule nucleating/elongation components such as \(\gamma\)-tubulin, Nedd1 (unpublished data), or EB1 (Fig. 6 C) at spindle poles during mitosis in response to MISP siRNA treatment. Furthermore, microtubule regrowth activity after MISP knockdown was similar as in the control cells after cold treatment (Fig. S4 D), ruling out microtubule nucleation problems. As MISP mainly localizes at the cell cortex and does not bind to microtubules directly, as shown in Fig. S5 C, we speculate that MISP regulates the stability of astral MTs probably through disturbing their binding to microtubule stabilizers at the tip of astral MTs. Thus, the identification of the linker protein(s) which regulate(s) the function of MISP on astral MTs will be of future interest and will help to understand the mitotic functions of MISP in detail.

Interestingly, we found that the role of MISP in both mitotic progression and spindle positioning is positively regulated through Plk1-dependent phosphorylation. A likely involvement of Plk1 in the regulation of spindle orientation has been demonstrated in two recent studies. Plk1 negatively regulates cortical dynein localization downstream of LGN-NuMA by controlling the interaction between dynein–dynactin and LGN–NuMA (Kiyomitsu and Cheeseman, 2012). In addition, Plk1-dependent phosphorylation of Dvl2, a Wnt signaling component that localizes to the spindle and spindle poles, regulates spindle orientation (Kikuchi et al., 2010). In this study, we show that Plk1 phosphorylation on MISP negatively regulates the cortical recruitment of p150\textsubscript{fluid}. Because the phospho-mimicking MISP 6DP mutant but not 7AP or 9AC was able to complement the distribution of p150\textsubscript{fluid} at the cortex (Fig. 7, E and F), Plk1 phosphorylation contributes to the function of MISP in this process, although it does not seem to be mediated by the MISP–p150\textsubscript{fluid} interaction (Fig. S5 F). We speculate that the mechanism on how MISP regulates cortical localized p150\textsubscript{fluid} could be a consequence of its impact on astral MTs, which facilitate the microtubule minus end (spindle pole)–directed transport of the cortical localized

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Figure 7. **MISP associates with p150glued and regulates its distribution at the cortex.** (A) p150<sup>glued</sup> was immunoprecipitated from nocodazole-blocked HeLa cells and analyzed by Western blot with indicated antibodies. Asterisk, unspecific recognized band in the control mouse IgG immunoprecipitate. (B) MTs were pelleted by high-speed centrifugation after nocodazole or taxol incubation. Input (I), supernatant (S), and the pellet (P) were analyzed by Western blot with indicated antibodies. (C) Z-stack images (0.4 µm per stack) of p150glued in metaphase HeLa cells depleted with MISP or control siRNA after cytoplasmic extraction. Right panel shows images with x-z projection. Arrowheads show the cortical localized p150glued. (D) Quantification of the percentage of metaphase cells with cortical accentuated and distributed p150<sup>glued</sup>; n = 50 cells each from three independent experiments. (E) HeLa cells transfected with control or MISP siRNA, or MISP siRNA under expression of GFP or siRNA-resistant versions of GFP-MISP WT/6DP/7AP/9AC were stained for p150<sup>glued</sup>. Representative images with z-projection are shown. Arrowheads indicate the cortical localized p150<sup>glued</sup>. (F) Quantification of the percentage of metaphase cells with cortical accentuated and distributed p150<sup>glued</sup>; n = 50 cells each from three independent experiments. Bars: (C and E) 10 µm. Error bars in D and F represent SD. Student’s t test was used to calculate p-value for comparison of control and experimental samples.
were grown in LB medium at 30°C and protein expression was induced. All recombinant proteins were expressed in pCMV-3Tag-1. The Plk1 PBD was also PCR amplified and cloned into the BamHI–XhoI sites of pCMV-3Tag-1. MISP fragments were PCR amplified and cloned into the XhoI–BamHI sites of the pEGFP-C1 (Takara Bio Inc.), the BamHI–XhoI sites of pGEX-4T3 (GE Healthcare, Germany), PCR amplified and cloned into the XhoI–BamHI sites of the pCMV-3Tag-1 (Agilent Technologies). All MISP fragments were transfected and plasmid DNA transfections, Hela cells were transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions.

Antibodies
A rabbit polyclonal antibody was raised against the full-length GST-MISP protein that was expressed as a GST fusion in Escherichia coli [Innova- gen]. The antibody was purified using the corresponding protein immobi- lized on CNBr-activated Sepharose. Other antibodies used in this study: mouse anti-Plk1 (F8 and 36–298), mouse anti-CDk1 (p34), and rabbit anti-GST (Z5), purchased from Santa Cruz Biotechnology, Inc.; rabbit anti- cyclin B1 was generated against full-length human Cyclin B1 purified from E. coli as described previously (Hoffmann et al., 1993); mouse anti-Flag M2, mouse anti-BubR1 (B8), mouse anti-Anillin/FITC conjugate, mouse anti-γ-tubulin (GTU-88), and mouse anti-α-tubulin were obtained from Sigma-Aldrich; mouse anti-actin (LA20) was from EMD Millipore; mouse anti-phospho-H3 Ser10, mouse anti-PARP, mouse anti-actin (C4), and mouse anti-EB1 were from BD; rabbit anti-Mad2 (PRB452C) was obtained from Covance; rabbit anti-GFP antibody was obtained from Novus Biologics; rabbit anti-phosphohistone H3 Ser10 (6G3) and rabbit anti-MEK1/2 were from Cell Signaling Technology; rabbit anti-pericentrin from A. Merdes (CNRS, Toulouse, France); rabbit anti-Cep135 from R. Kuriyama (University of Minnesota, Minneapolis, MN). Mouse anti-CENP-E (1H12) was from Abcam; mouse anti-dynein intermediate chain (IC) was purchased from Sigma-Aldrich (clone 70.1). Peroxidase-conjugated donkey anti–rabbit and goat anti–mouse antibodies were purchased from Jackson Immunoresearch Laboratories, Inc.; Alexa Fluor 488 and 594 anti–mouse and Alexa Fluor 488 and 594 anti–rabbit were purchased from Invitrogen. Atto 488 Phalloidin was purchased from Sigma-Aldrich.

Molecular cloning
MISP (DKFZp686H18209) was received from S. Wiemann (DKFZ, Heidelberg, Germany), PCR amplified and cloned into the XhoI–BamHI sites of the pEGFP-C1 (Takara Bio Inc.), the BamHI–XhoI sites of pGEX-4T3 (GE Healthcare), and of pCMV-3Tag-1 (Agilent Technologies). All MISP fragments were PCR amplified and cloned into the BamHI–XhoI sites of pCMV-3Tag-1. pRCMV-Plk1 was received from S. Lev (Weizmann Institute, Rehovot, Israel), PCR amplified and cloned into the EcoRI–XhoI sites of pGEX-4T1 and the EcoRI–XhoI sites of pCMV-3Tag-1. The Plk1 PBD was also PCR amplified and cloned into the EcoRI–XhoI sites of pGEX-4T1. pEGFP-E3B (pEGFPN1; Takara Bio Inc.) was a kind gift from L. Wordeman (University of Washington, Seattle, WA, Stepanova et al., 2003).

Recombinant protein expression
All recombinant proteins were expressed in E. coli BL21 (DE3). Bacteria were grown in LB medium at 30°C and protein expression was induced with 1 mM isopropyl-β-D-thiogalactoside for 16 h at 18°C. MISP WT/ΔDP/ 7AP/9AC and the Plk1 PBD carrying an N-terminal GST tag were natively purified by single-step affinity chromatography using GST- Sepharose according to the manufacturer’s instructions.

Interaction experiments and kinase assays
For communoprecipitations, 1–8 mg of cell lysate were incubated with 1–7 μg of specific antibodies in NP-40 buffer (40 mM Tris, pH 7.5, 150 mM NaCl, 0.5% NP-40, 0.5 mM EDTA, 10 mM β-glycerophosphate, and 5 mM NaF) or RIP buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% NP-40, and 0.5% deoxycholate) for 4 h at 4°C and collected by addition of 10 μl protein G-Sepharose (GE Healthcare). After extensive washing, the beads were denatured at 95°C in 2X SDS buffer and analyzed by SDS-PAGE and Western blot. For Western ligand blots were performed in TBST (50 mM Tris-HCl, pH 7.4, 137 mM NaCl, 0.1% Tween 20, and 4% [wt/vol] skim milk powder) as described previously (Neef et al., 2003). Flag-vector or Flag-MISP WT/phospho-mutant transfected Hela cells lysates were sepa- rated on SDS-PAGE followed by blotting on a nitrocellulose membrane. Bacterially purified GST-PBD or GST-FAM was incubated with the mem- brane, respectively. Bound proteins were detected using rabbit antibodies to GST and mouse anti-Flag M2 antibodies, respectively.

For in vitro pull-down experiments, GST-Plk1 or GST-MISP was im- mobilized on GST-Sepharose beads and incubated for 3 h at 4°C with mi- totic Hela lysates in pull-down buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 10 mM β-glycerophosphate, 5 mM NaF, 5 mM MgCl2, and 5% glycerol). Beads were washed three times with pull-down buffer and eluted once with pull-down buffer + 50 mM NaCl and once with pull-down buffer + 850 mM NaCl. After TCA precipitation, bound proteins were analyzed by SDS-PAGE and Western blot or silver staining followed by subsequent mass spectrometry.

To assay Plk1 and Cdk1 kinase activities, His-Plk1 (EMD Millipore), or GST-Cyclin B/His-Cdk1 (EMD Millipore) was incubated with bacterially purified recombinant GST-MISP wt/7AP/9AC as substrates for 30 min at 30°C in kinase assay buffer (50 mM Tris, pH 7.5, 5 mM MgCl2, and 1 mM DTT) supplemented with 0.5 μM ATP and 2.5 μl of γ[32P]ATP (PerkinElmer), and analyzed by autoradiography after SDS-PAGE. Kinase activities were inhibited by BI2536 (Boehringer Ingelheim) and roscovitine (Sigma-Aldrich) for Plk1 and Cdk1, respectively.

siRNAs
siRNAs used in this study were directed against the following sequences: MISP O1, 5′-ACUGCGUGUCAGAGUCUCCUCCUUC3; MISP O2, 5′-GUGUC-AACUGUGUGGAGAAGAG3′; Plk1, 5′-GGGGCGCUGGUGAAGC3′; Mad2, 5′-UGCAAAGAACUUCAACUUGGCGG3′; firefly luciferase 5′Q (control), 5′-CUUCCGCGGAAUACUUGCGG3′; CENP-E, 5′-AACGG-GAUGUGGUGACUCCUC3′; p150(20), 5′-GAAGCAGAGCAAGUAAU- U3′. MISP O11 was purchased from Invitrogen and Mad2 siRNA was a gift from O. Gruss (ZMBH, Heidelberg, Germany). All other siRNAs were purchased from Applied Biosystems.

Immunofluorescence microscopy and live-cell imaging
Cells grown on coverslips were either fixed with ice-cold methanol for 10 min at −20°C, or with 3.7% formaldehyde at RT for 10 min or with 4% paraformaldehyde at RT for 10 min. For cytoplasmic extraction, cells were pretreated with PHEM buffer (60 mM Pipes, pH 6.9, 25 mM Heps, 10 mM EGTA, 2 mM MgCl2, 0.5% Triton X-100, and 10 μM taxol) for 1 min at RT. They were washed with PBS and blocked with 3% BSA/PBS for 30 min. Cells were then incubated with primary antibodies for 2 h and with Alexa Fluor 488– or Alexa Fluor 594–conjugated secondary antibodies for 1 h at RT. DNA was stained with Hoechst 33342 (Molecular Probes). Cells were washed three times with PBS in between antibody incubations at RT. Coverslips were mounted in Mowiol 4:88 mounting medium. Images were taken with a spinning disk confocal system (UltraView ERS-5; PerkinElmer) and 100X NA 1.40 oil immersion objective on an inverted microscope [TI; Nikon] connected to an electron multiplying charge-coupled device camera (Hamamatsu Photonics). Fluorescence intensity was analyzed using ImageJ software (National Institutes of Health). Relative astral MT intensities were calculated within the equatorial stack defined by spindle pole maximum intensity using ImageJ software to create 8-bit RGB TIFF files. Images were then processed with Photoshop CS3 (Adobe Systems) to produce the figures.

For live-cell imaging experiments, Hela Kyo RFP-α-tubulin/RFP- H2B cells were grown in a Lab-Tek II chambered coverglass (8 chamber) for 24 h after siRNA transfection and were monitored afterward by a TIRF 40X NA 1.3 oil immersion objective [Nikon] on an inverted microscope (TI; Nikon) connected to an electron multiplying charge-coupled device camera (Xenon DU897; Andor Technology) at 5°C, CO2, 37°C, 90% humidity.
Lasers of 488- and 561-nm wavelength were used for fluorochrome excitation. Multi-point images were taken with 9 z-stacks spaced 2 µm apart every 6 min for 8 h by NIS-Elements software (Nikon) and were deconvoluted using Huygens deconvolution software (Scientific Volume Imaging). HeLa Kyoto GFP-H2B cells were grown in a Lab-Tek II chambered coverglass (4 chamber) for 28 h after siRNA transfection and were monitored afterward by a spinning disc confocal system (UltraView ERS-VoX, PerkinElmer) with an inverted microscope (Ti; Nikon) with a 40× NA 1.30 oil immersion objective at 5% CO2, 37°C, 90% humidity. Multi-point images were taken with 9 z-stacks spaced 2 µm apart every 8–12 h by Volocity software. Phase-contrast images were taken with one z-stack in the middle position. For the EB3 tracking experiment, GFP-EB3 was transiently transfected into HeLa cells followed by MISP siRNA treatment for 48 h. 7 planes spaced apart by 2 µm were captured with the maximum rate of 1 min (60x NA 1.30 oil immersion objective) by Volocity software. Maximum intensity projection of the fluorescent channels was performed by ImageJ software to create 8-bit RGB TIFF files and movies.

Spindle positioning assay
HeLa cells plated on fibronectin-coated coverslips were transfected with control siRNA or MISP siRNA and fixed with 70% ice-cold ethanol and 0.5% NP-40 in PBS. Cells were resuspended in FACS buffer (38 mM NaCitrate and 1 µg/ml RNase A) on a FACSort. FITC and 50 µg/ml propidium iodide for 10 min at RT before analysis.

Multi-point images were taken with an inverted microscope (Ti; Nikon) with a 40× NA 1.30 oil immersion objective by a spinning disk confocal system (UltraView ERS-VoX; PerkinElmer). Kyoto GFP-H2B cells were grown in a Lab-Tek II chambered coverglass using Huygens deconvolution software (Scientific Volume Imaging). HeLa cells were fixed with 70% ice-cold ethanol and 0.5% NP-40 in PBS. Cells were resuspended in binding buffer (10 mM Hepes, pH 7.5, 140 mM NaCl, 1.5 mM MgCl2, and 0.5% NP-40), incubated at 4°C for 15 min, and sonicated four times for 15 s each. Cleared lysates were resuspended in 1× SDS buffer and analyzed by SDS-PAGE. MAPF (MT-associated protein fraction containing 60% MAP2 and 40% tau proteins) was included in the kit as a positive control.

MT cosedimentation assay
Mitotic HeLa cells were resuspended in PEM buffer (80 mM Pipes, pH 6.9, 1 mM EGTA, 1 mM MgCl2, and 0.5% NP-40). Cells were incubated with 10 µM taxol or nocodazole (negative control), 1 mM GTP, for 15 min, and sonicated four times for 15 s each. Cleared lysates were resuspended in 1× SDS buffer and analyzed together with the supernatant by SDS-PAGE. Mitotic HeLa cells were resuspended in PEM buffer (80 mM Pipes, pH 6.9, 1 mM EGTA, 1 mM MgCl2, and 0.5% NP-40), incubated at 4°C for 1.5 h using a TLA100.3 rotor (Beckman Coulter) at 24°C. The pellet was resuspended in 0.2 volumes PEM buffer + 10 µM nocodazole (negative control) 1 rpm, 1 mM AMP-PNP, and 8% 2-methyl-2,4-pentanediol (Sigma-Aldrich) for 30 min at 30°C and layered over a 25% glycerol cushion in PEM buffer. After centrifugation at 48,000 rpm in a TLS55 rotor (Beckman Coulter), the resulting pellets were resuspended in 0.2 volumes PEM buffer + 10 µM taxol or nocodazole and were recentrifuged as described above. The final MT pellet was resuspended in 1× SDS buffer and analyzed by SDS-PAGE and Western blot together with supernatant.

MT regrowth assay
MTs were depolymerized by cold treatment for 30 min at 4°C. Repolymerization of MTs was then achieved by addition of prewarmed medium and a temperature shift back to 37°C for 0, 1, and 3 min.

FACS analysis
For cell cycle analysis, cells were fixed with 70% ice-cold ethanol and DNA content was analyzed by propidium iodide staining (50 µg/ml) in FACS buffer (38 mM NaCitrate and 1 µg/ml RNase A) on a FACS sort cytometer (BD). For annexin staining, AnnexinV-FITC (Sigma-Aldrich) was used as described in the manufacturer’s procedure. In brief, cells were resuspended in binding buffer (10 mM Hepes, pH 7.5, 140 mM NaCl, and 2.5 mM CaCl2) and incubated with 25 µg/ml AnnexinV-FITC and 50 µg/ml propidium iodide for 10 min at RT before analysis on the FACSsort.

Computational sequence analysis and comparison of MISP
The initial sequence similarity searches were performed with BLAST (Altschul et al., 1997) against the nonredundant protein sequence database Uniref50 (Wu et al., 2006). Multiple sequence alignments of homologous protein sequences were generated with the program T-Coffee (Notredame et al., 2000) using default parameters that were slightly refined manually. Profiles of the alignment as global hidden Markov models (HMMs) were generated using HMMer (Eddy, 1995). Iterative similarity searches (E-value inclusion threshold < 0.005), using HMMer (Eddy, 1995), with the repeated region identified in the three vertebrate paralogues (MISP, AKAP2, and LOC1113230, UniProt: Q8IVT2_HUMAN, Q9Y2DS_HUMAN, Q96F77_HUMAN, respectively) as query identified remote homologues including: urochordates (Ciona intestinalis and Ciona freshwater), hemichordates (Saccoglossus kowalevskii), echinoderms (Strongylocentrotus purpuratus), cnidarians (Nematostella vectensis), mollusks (Lotta gigantea and Crassostrea gigas), annelids (Capitella teleta and Helobdella robusta), and arthropods (Daphnia pulex, Anopheles gambiae, and, Ixodes scapularis, among others), including the Drosophila melanogaster CG2556 protein, UniProt: Q9VVM2_DROME. Sequences were obtained from UniProt, JGI (Joint Genome Institute), NCBI-GenBank, and NCBI-blast but were supplemented by manually assembled ESTs and FGENES+ predicted gene models (Solovyev et al., 2006).

Online supplemental material
Fig. S1 shows the identification and characterization of MISP, and its binding activity to Plk1. Fig. S2 shows the identification of the mitotic phosphorylation sites on MISP and the localization of Flag-MISP WT and phospho-mutants in HeLa cells. Fig. S3 shows that MISP is required for mitotic progression. Fig. S4 shows that MISP knockdown leads to mitotic defects and apoptosis. Fig. S5 shows that MISP does not directly bind to MTs and associates with p150glued in a phosphorylation-independent manner. Videos 1 and 2 show mitotic progression in MISP siRNA-treated GFP-α-tubulin/FH2B HeLa cells. Video 3 shows mitotic progression in control siRNA-treated GFP-α-tubulin/FH2B HeLa cells. Video 4 shows mitotic progression in MISP siRNA-treated GFP-FH2B HeLa cells. Video 5 shows mitotic progression in control siRNA-treated GFP-FH2B HeLa cells. Video 6 shows spindle microtubule dynamics tracked by GFP-FB3 in control siRNA-treated HeLa cells. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201207050/DC1. Additional data are available in the JCB DataViewer at http://dx.doi.org/10.1083/jcb.201207050.dv.

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Figure S1. **Identification and characterization of MISP and its binding activity to Plk1.** (A) Plk1 immunoprecipitated from HeLa cells arrested in late mitotic stages by nocodazole block and release for 1.5 h was subjected to an in vitro kinase assay. Indicated bands were sent for mass spectrometry analysis. (B) Depicted proteins from mass spectrometry analysis are shown with MASCOT score, molecular weight, and coverage. (C) Computational analysis of MISP in different species and homologies with human MISP. Schematic representation of conserved domains in the MISP family and multiple sequence alignment of the conserved regions of representative proteins of different species. The coloring scheme indicates average BLOSUM62 score (correlated with amino acid conservation) in each alignment column: red (greater than 3), violet (between 3 and 1.5), and light yellow (between 1.5 and 0.5). (D) HeLa cells were transfected with control or MISP siRNA (Ol1/Ol2) for 48 h. Cell lysates were analyzed by Western blot. (E) Western blot analysis of a GST pull-down assay using lysates from mitotic HeLa cells expressing Flag-MISP and bacterially purified GST-Plk1 PBD, FAM, or GST as bait. (F) Western blot analysis of a GST pull-down assay using lysates from mitotic HeLa cells expressing Flag-MISP full length (FL) or indicated truncations, and bacterially purified GST-Plk1 PBD, FAM, or GST as bait. (G) Schematic overview of MISP FL and truncations and their binding activities to Plk1.
Figure S2. Identification of the mitotic phosphorylation sites on MISP and localization of Flag-MISP WT and phospho-mutants. (A) Cdk1 was immunoprecipitated from mitotically arrested HeLa cell lysates and Western blot analysis was performed. (B) GST-MISP or GST alone was incubated with GST-cyclin B/His-Cdk1 (in the presence or absence of 5 µg/ml of the Cdk inhibitor roscovitine) and analyzed by autoradiography. (C) Phospho-peptides in MISP were identified by mass spectrometry analysis from in vitro kinase assays using His-Plk1 or GST-cyclin B/His-Cdk1. The possible phosphorylation sites for Plk1 (red) and for Cdk1 (green) were mutated to alanine or aspartic acid. For the MISP mutants of Plk1 phosphorylation sites, S471 has additionally been included into the analysis based on the mass spectrometry analyses of the Plk1-dependent phosphoproteome (Santamaria et al., 2011). S382 has been excluded from the phospho-mimicking mutant (6DP) due to technical constraints. (D) HeLa cells transfected with GFP-MISP WT/7AP/9AC were analyzed by Western blot with indicated antibodies with and without nocodazole treatment. Asterisk indicates highest phosphorylated band with slowest migrating form of GFP-MISP. (E) HeLa cells transfected with Flag-MISP in the presence or absence of Cyto. D [2 µM, 10 min]. Cells were fixed and stained with Flag antibody (red) and Atto 488 Phalloidin (green). Bars, 10 µm. Insets show higher magnification of the framed regions. Bars, 5 µm. (F) Representative images of mitotic HeLa cells transfected with Flag-MISP WT, 6DP, 7AP, or 9AC. Flag-MISP (red), Atto 488 Phalloidin (green), DNA (blue). Bar, 10 µm.
Figure S3. **MISP is required for mitotic progression.** (A and B) A representative image of a mitotic HeLa cell with co-stained MISP (red), α-tubulin (A, green) or Plk1 (B, green), and Hoechst 33342. (C) HeLa cells transfected with control or MISP siRNA were stained with MISP antibody (red), Atto 488 Phalloidin (green), and Hoechst 33342. (D) HeLa cells transfected with GFP alone or an siRNA (Ol2)-resistant version of GFP-MISP were treated with MISP or control siRNA for 48 h. The mitotic index was evaluated by α-tubulin and p-Ser10 H3 staining. n = 200 cells from three independent experiments. (E) Lysates prepared from cells from D were analyzed by Western blot with indicated antibodies. The dividing white lane marks the grouping of images from different parts of the same gel, as an intervening lane was removed for presentation purposes. (F) HeLa cells were transfected with control or MISP siRNA for 48 h, and stained with BubR1 (green), CREST (red), and Hoechst 33342. (G) Quantification of the mitotic index of HeLa cells transfected with MISP or control siRNA and in combination with Mad2 siRNA for 48 h. n = 200 cells from three independent experiments. (H) Lysates prepared from cells from G were analyzed by Western blot with indicated antibodies. Bars: (A, B, C, and F) 10 µm. Error bars in D and G represent SD. Student’s t test was used to calculate p-value for comparison of control and experimental samples.
Figure S4. **MISP knockdown leads to mitotic defects and apoptosis.** (A and B) MISP or control siRNA-treated HeLa cells were stained with α-tubulin (green) and pericentrin (A, red) or α-tubulin (green) and Cep135 (B, red). (C) Quantification of the percentage of HeLa cells in metaphase with disrupted poles in MISP or control siRNA and in combination with Mad2 siRNA-treated cells (48 h after transfection). n = 200 cells from three independent experiments. (D) MISP or control siRNA-transfected HeLa cells were exposed at 4°C for 30 min and shifted back to 37°C for indicated time points. Cells were stained with α-tubulin (green) and β-tubulin (red). (E) Scatter plots showing spindle angles from the spindle orientation assay described in Fig. 5, C and D, with calculated mean value. n = 150 cells from three independent experiments. (F) Quantification of the percentage of metaphase cells with indicated spindle angles with MG132 treatment for 2 h before fixation. n = 50 cells were calculated from a single experiment. (G) HeLa cells transfected with control or CENP-E siRNA were stained with CENP-E antibody (green), CREST (red), and Hoechst 33342. (H) Control or MISP siRNA-treated HeLa cell lysates were analyzed by Western blot with indicated antibodies. (I) HeLa cells treated with MISP or control siRNAs for 48 h were stained with propidium iodide and AnnexinV-FITC to analyze the DNA content and the apoptotic cells by FACS, respectively (left panel). Quantification of gate R1 of the AnnexinV-FITC–positive cells from three independent experiments was shown in the right panel. Bars: (A, B, D, and G) 5 µm. Error bars in C, E, and I represent SD. Student's t test was used to calculate p-value for comparison of control and experimental samples.
Figure S5. **MISP does not directly bind to MTs and associates with p150\textsuperscript{glued} in a phosphorylation-independent manner.** (A) Quantification of the measured spindle intensity ($I_{\text{spindle}}$) as described in Fig. 6 B; $n = 60$ cells from three independent experiments. Error bars represent SD. Student’s $t$ test was used to calculate $p$-value for comparison of control and MISP siRNA-treated cells. (B) HeLa cells treated with MISP or control siRNA were stained with $\alpha$-tubulin (green) and CREST (red) after cold treatment for 10 min at 4°C before fixation. Bar, 10 $\mu$m. Enlargements of the framed insets are shown on the right. Bar, 2.5 $\mu$m. (C) In vitro MT-binding assay using polymerized MT and GST (negative control), GST-MISP, or MAPF (MT-associated protein fraction containing 60% MAP2 and 40% tau proteins, positive control). P, pellet; S, supernatant. (D) GST pull-down was performed using GST-MISP and mitotic HeLa cell lysate and analyzed by mass spectrometry. Candidate binding proteins are shown with MASCOT score, molecular weight, and coverage. (E) Taxol-stabilized MTs were pelleted by high-speed centrifugation in control, MISP, or p150\textsuperscript{glued} siRNA-treated mitotic HeLa cell lysates. Input (I), supernatant (S), and pellet (P) were analyzed by Western blot with indicated antibodies. (F) GST pull-down assays were performed using bacterially purified GST, GST-MISP WT, 6DP, 7AP, or 9AC and mitotic HeLa cell lysates. Ponceau S staining image and Western blot analyses with indicated antibodies were shown.
Video 1.  **Mitotic progression in MISP siRNA-treated HeLa GFP-α-tubulin/RFP-H2B cells.** HeLa GFP-α-tubulin/RFP-H2B cells were transfected with MISP siRNA and 24 h later analyzed by time-lapse confocal microscopy using a laser scanning microscope (Ti TIRF; Nikon). Frames were taken every 6 min for 246 min.

Video 2.  **Mitotic progression in MISP siRNA-treated HeLa GFP-α-tubulin/RFP-H2B cells.** HeLa GFP-α-tubulin/RFP-H2B cells were transfected with MISP siRNA and 24 h later analyzed by time-lapse confocal microscopy using a laser scanning microscope (Ti TIRF; Nikon). Frames were taken every 6 min for 282 min.

Video 3.  **Mitotic progression in control siRNA-treated HeLa GFP-α-tubulin/RFP-H2B cells.** HeLa GFP-α-tubulin/RFP-H2B cells transfected with control siRNA and 24 h later analyzed by time-lapse confocal microscopy using a laser scanning microscope (Ti TIRF; Nikon). Frames were taken every 6 min for 54 min.

Video 4.  **Mitotic progression in MISP siRNA-treated HeLa GFP-H2B cells.** HeLa GFP-H2B cells were transfected with MISP siRNA and 28 h later monitored by a spinning disk microscope (UltraView ERS-VoX; PerkinElmer). Frames were taken every 3 min for 474 min.

Video 5.  **Mitotic progression in control siRNA-treated HeLa GFP-H2B cells.** HeLa GFP-H2B cells were transfected with control siRNA and 28 h later monitored by a spinning disk microscope (UltraView ERS-VoX; PerkinElmer). Frames were taken every 3 min for 474 min.
Video 6. **EB3 dynamics in MISP siRNA-treated HeLa cells.** HeLa cells were transfected with GFP-EB3 and MISP siRNA and 48 h later monitored by a spinning disk microscope [UltraView ERS-VoX; PerkinElmer]. Frames were taken every 2 s for 1 min.

Video 7. **EB3 dynamics in control siRNA-treated HeLa cells.** HeLa cells were transfected with GFP-EB3 and control siRNA and 48 h later monitored by a spinning disk microscope [UltraView ERS-VoX; PerkinElmer]. Frames were taken every 2 s for 1 min.

Reference