Release of Mps1 from kinetochores is crucial for timely anaphase onset

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Mps1 kinase activity is required for proper chromosome segregation during mitosis through its involvements in microtubule–chromosome attachment error correction and the mitotic checkpoint. Mps1 dynamically exchanges on unattached kinetochores but is largely removed from kinetochores in metaphase. Here we show that Mps1 promotes its own turnover at kinetochores and that removal of Mps1 upon chromosome biorientation is a prerequisite for mitotic checkpoint silencing. Inhibition of Mps1 activity increases its half-time of recovery at unattached kinetochores and causes accumulation of Mps1 protein at these sites. Strikingly, preventing dissociation of active Mps1 from kinetochores delays anaphase onset despite normal chromosome attachment and alignment, and high interkinetochore tension. This delay is marked by continued recruitment of Mad1 and Mad2 to bioriented chromosomes and is attenuated by Mad2 depletion, indicating chronic engagement of the mitotic checkpoint in metaphase. We propose that release of Mps1 from kinetochores is essential for mitotic checkpoint silencing and a fast metaphase-to-anaphase transition.

Introduction

To prevent chromosome missegregations, the onset of anaphase is inhibited by coordinated actions of the error correction and mitotic checkpoint machineries until all chromosomes have stably bioriented. The mitotic checkpoint directs formation of a mitotic checkpoint complex, which is catalyzed on unattached kinetochores and inhibits the anaphase-promoting complex/cyclosome (APC/C; for review see Musacchio and Salmon, 2007). As soon as all kinetochores have attached to microtubules in a stable fashion, the mitotic checkpoint is silenced and inhibition of APC/C is released, ultimately causing anaphase initiation and mitotic exit (for review see Musacchio and Salmon, 2007). Checkpoint silencing in human cells requires dynein-mediated removal of Spindly–RZZ–Mad1/Mad2 from attached kinetochores (Howell et al., 2001; Barisic et al., 2010; Gassmann et al., 2010), p31comet-mediated inhibition of Mad2 conformational activation (Xia et al., 2004; Mapelli et al., 2006), and APC/C-assisted disassembly of the inhibitory complex (Reddy et al., 2007).

The kinase Mps1 is an important player in prevention of chromosomal instability (Jelluma et al., 2008b; Tighe et al., 2008), as its activity is crucial for chromosome biorientation by promoting attachment error correction as well as for APC/C inhibition by the mitotic checkpoint. In human cells, Mps1 regulates error correction (Jelluma et al., 2008b; Santaguida et al., 2010; Sliedrecht et al., 2010) by enhancing Aurora B activity through direct phosphorylation of Borealin (Jelluma et al., 2008b; Bourhis et al., 2009; Kwiatkowski et al., 2010; Sliedrecht et al., 2010), and may in addition use other mechanisms (Espeut et al., 2008; Maciejowski et al., 2010; Santaguida et al., 2010). Mitotic checkpoint regulation by Mps1 has been observed in many model systems (Hardwick et al., 1996; Weiss and Winey, 1996; He et al., 1998; Abrieu et al., 2001; Fisk and Winey, 2001; Stucke et al., 2002; Liu et al., 2003; Fischer et al., 2004; Jelluma et al., 2008b), and its enzymatic activity, at least in humans, directs a number of checkpoint proteins including Mad1 to unattached kinetochores (see Lan and Cleveland, 2010 for a

Supplemental material can be found at:
http://doi.org/10.1083/jcb.201003038
recent summary), allows Mad2 conformational activation (Hewitt et al., 2010), and stabilizes the cytoplasmic APC/C inhibitory complex(es) (Maciejowski et al., 2010).

Mps1 activity rises during mitosis (Stucke et al., 2002), at which time Mps1 dynamically localizes to kinetochores (Howell et al., 2004), dimerizes (Hewitt et al., 2010), and auto-activates by cross-phosphorylation of its activation loop (Kang et al., 2007; Mattison et al., 2007; Jelluma et al., 2008a). The underlying mechanisms of Mps1 kinetochore recruitment and dynamics, however, remain elusive. Mps1 requires the Hecl component of the microtubule-binding NDC80 complex to reach kinetochores (Martin-Lluesma et al., 2002; Meraldi et al., 2004), likely through a localization signal intrinsic to its N-terminal 300 amino acids that are also required for mitotic checkpoint function (Liu et al., 2003). Interestingly, a mutant lacking the N-terminal 100 amino acids also doesn’t reach kinetochores but still supports a mitotic checkpoint in cells that also express full-length, inactive Mps1 (Maciejowski et al., 2010). GFP-Mps1 only transiently associates with prometaphase kinetochores in PtK2 cells, and this association decreases as chromosomes establish attachments, reaching its lowest levels after chromosomes have aligned on the metaphase plate (Howell et al., 2004). We here address the regulation of Mps1 levels at kinetochores and investigate the reason for its fast turnover at these sites.

Results and discussion

Mps1 auto-regulates its dissociation from kinetochores

Mps1 exchanges on kinetochores during mitosis in PtK2 cells, showing monophasic recovery of 99% with a half-life of 9 s (Howell et al., 2004). To investigate the role of Mps1 kinase activity in recruitment and release of Mps1 at kinetochores, kinetochore levels of active and inactive Mps1 were examined by immunofluorescence. As noted by others (Hewitt et al., 2010), exogenous kinase-dead (KD, D664A) Mps1 (LAP-Mps1-KD) was found at much higher levels on unattached kinetochores of cells depleted of endogenous Mps1 than its active, wild-type (WT) counterpart (LAP-Mps1-WT; Fig. 1 A, Fig. S1 A). Short-term chemical inhibition of endogenous Mps1 or LAP-Mps1 with the specific inhibitor Mps1-IN-1 (Kwiatkowski et al., 2010) in HeLa cells, or of analogue-sensitive Mps1 (Mps1-as) with 23dMB-PP1 in U2OS-derived cells (Sliedrecht et al., 2010) corroborated this, causing a two- to tenfold increase in kinetochore-bound Mps1 (Fig. 1 B; Fig. S1, B and C). This is in excellent agreement with a recent study using another Mps1 inhibitor, AZ3146 (Hewitt et al., 2010). Together, these results show that the levels of Mps1 at kinetochores in prometaphase increase when Mps1 kinase activity is impaired.

We next addressed what substrates of Mps1 could affect Mps1 localization to kinetochores. Mps1 modifies itself in trans and in cis by autophosphorylation (Kang et al., 2007; Mattison et al., 2007; Jelluma et al., 2008a; Hewitt et al., 2010), phosphorylates the inner-centromere protein Borealin (Jelluma et al., 2008b; Bourhis et al., 2009; Sliedrecht et al., 2010), and likely phosphorylates many kinetochore-localized proteins (Espeut et al., 2008; and see Lan and Cleveland, 2010 for review of kinetochore proteins affected by Mps1 activity). Borealin phosphorylation and subsequent increase in Aurora B activity was not involved, as addition of the Aurora B inhibitor ZM447439 (Ditchfield et al., 2003) did not cause mislocalization of active Mps1 (Fig. S1, D and E). Interestingly, however, LAP-Mps1-KD levels at kinetochores were increased twofold upon depletion of endogenous Mps1 by RNAi compared with control (Fig. 1 C). This increase could be reduced by coexpression of LAP-Mps1-WT, which was, in turn, prevented by Mps1-IN-1 (Fig. 1 D). These results indicate that LAP-Mps1-KD could be displaced from kinetochores by the action of endogenous or exogenous active Mps1. A similar influence of Mps1 activity on its localization was observed when Mps1 was artificially targeted to peroxisomes via a C-terminal peroxisomal targeting sequence (PTS1; Gould et al., 1989). Only inactive Mps1-PTS1 was found in peroxisomes of interphase cells but its localization was prevented by coexpression of active Mps1-PTS1 (Fig. S1 F). These data thus show that Mps1 activity could also regulate its own localization when artificially targeted to a different location in a different phase of the cell cycle. Although the mechanism of auto-regulation at peroxisomes might be different from what happens on kinetochores, structural rearrangement of Mps1 by in trans autophosphorylation could account for delocalization from both sites.

Inhibition of Mps1 increases its residence time at kinetochores

Because short-term inhibition of Mps1 had no overt effects on its total cellular protein levels (Fig. S1 A), we examined if a decreased exchange rate at unattached kinetochores upon inactivation was causing Mps1 to accumulate at these sites by measuring FRAP. After photobleaching, LAP-tagged Mps1-as (Sliedrecht et al., 2010) recovered rapidly to 99% (Fig. 1 E). Addition of 23dMB-PP1 caused Mps1 half-life at kinetochores to increase ~1.5-fold (95% confidence intervals: uninhibited 0.88–1.36; inhibited 1.38–1.67 s) and recovery was reduced to 94% (Fig. 1 E; see Materials and methods for more details). Similar exchange and recovery kinetics of inhibited Mps1 was observed on kinetochores of PtK2 cells (Fig. S1 G). Although these analyses show a shorter Mps1 half-time of recovery at kinetochores than previously reported (Howell et al., 2004), technical limitations (see Materials and methods) prohibited us from drawing conclusions on the absolute recovery times of LAP-Mps1 on kinetochores, but did allow comparison of kinetics between uninhibited and inhibited Mps1. The small but significant change in half-life and in the size of stable, nonexchange-pool of kinetochore-bound Mps1 may underlie the higher protein levels detected by immunofluorescence (Fig. 1, A and B; Fig. S1, B and C). Nevertheless, inactive Mps1 still had a high exchange rate at the kinetochore, indicating that the exchange of Mps1, although influenced by it, is not fully dependent on its kinase activity.

To examine in what circumstances Mps1 is recruited to kinetochores, cells were treated with Mps1-IN-1 but without spindle drugs, allowing accumulation of Mps1 on kinetochores of aligned and misaligned chromosomes that are nevertheless
Figure 1. Mps1 accumulates on kinetochores when inhibited. (A) Immunolocalization of LAP-Mps1 in U2OS cells cotransfected with Mps1 shRNA and LAP-Mps1-WT or LAP-Mps1-KD and treated with nocodazole and MG132. Immunoblot shows expression of LAP-Mps1-WT and -KD in whole-cell lysates. (B) Immunolocalization of Mps1 and Mad2 in HeLa cells treated as indicated. Graph represents quantitation of fluorescence intensities (±SEM, 5 cells per condition, 22 kinetochores/cell). (C and D) Quantitation of fluorescence intensities at kinetochores of U2OS cells transfected and treated as indicated (±SEM, 8 cells per condition, 22 kinetochores/cell). (E) UTRM-LAP-Mps1<sup>M602G</sup> cells were treated as indicated, and 0.81-µm<sup>2</sup> areas around single kinetochores (green squares in top panel) or in the cytoplasm (white squares) were bleached at t = 1 s. Graphs show average fluorescence intensities, shaded areas indicate SDs, and percentages indicate average recovery between 10 and 12 s. (F) Mps1 localization on bioriented and mono-oriented kinetochores in a HeLa cell treated as indicated.
attached (Jelluma et al., 2008b). Under this condition, Mps1 was present at kinetochores of misaligned chromosomes, but its levels were strongly reduced on bioriented chromosomes (Fig. 1 F). Inhibiting Aurora B prevented the accumulation of inhibited Mps1 on non-bioriented chromosomes (Fig. S1 H; Santaguida et al., 2010). Thus, recruitment of Mps1 to kinetochores is strongly diminished as soon as chromosomes have bioriented. In agreement with this, Mps1 levels diminish on attached prometaphase kinetochores of PtK2 cells compared with unattached prometaphase kinetochores, and diminish further on late metaphase kinetochores (Howell et al., 2004).

Combined, the data support a model in which Mps1 is recruited to non-bioriented chromosomes where it is rapidly released through a mechanism that involves Mps1-dependent phosphorylation. Upon biorientation Mps1 is no longer recruited, causing its depletion from kinetochores in metaphase cells.

Preventing dissociation of Mps1 from kinetochores prolongs metaphase

To address the functional relevance of rapid release of Mps1 in prometaphase and its depletion in metaphase, a pool of Mps1 was prevented from leaving the kinetochore by fusion to the Mis12 protein. Mis12 is a constitutive kinetochore protein in mitosis (Cheeseman et al., 2004; Obuse et al., 2004) and fusion to INCENP was previously shown to efficiently recruit INCENP/Aurora B to metaphase kinetochores (Liu et al., 2009). LAP-tagged Mis12-Mps1 (LAP-Mis12-Mps1-WT) was readily visible on kinetochores in prophase, prometaphase, and metaphase (Fig. 2 A) at a similar location as wild-type Mps1 (nocodazole-treated; Fig. S2 A). Mis12-Mps1 fully supported mitotic checkpoint activity in Mps1-depleted cells, showing the fusion did not prevent Mps1 functioning (Fig. 2 B). Please note that ∼60% of Mis12-Mps1 associated transiently with kinetochores (Fig. S2, B and C), indicating that this approach could not tether all cellular Mps1 to kinetochores and thus maintained essential cytoplasmic pools of Mps1 (Maciejowski et al., 2010). We speculate that Mps1 dimerization (Hewitt et al., 2010) and/or targeting via the Mis12-intrinsic kinetochore localization domain may have been responsible for recruitment and release of this exchanging pool of Mis12-Mps1. Nevertheless, a significant fraction (∼40%) of Mis12-Mps1 was stably associated with kinetochores (Fig. S2 B), allowing evaluation of the effect of sustained presence of Mps1 at kinetochores on chromosome segregation.

Strikingly, expression of Mis12-Mps1-WT but not -KD or LAP-Mps1-WT in U2OS cells caused pronounced extension of metaphase in more than 70% of cells, even up to 10 h in some cases (Fig. 2 C; Fig. S2, D and E). Treatment with Mps1-IN-1 showed that these prolonged metaphases were due to sustained Mps1 activity on kinetochores (Fig. 2 C). Removal of Mad2 by expression of Mad2 shRNA effectively prevented the delays in anaphase onset in cells expressing Mis12-Mps1, showing Mad2 and thus mitotic checkpoint activity was responsible for the delays (Fig. 2 D).

Sustained Mps1 activity at kinetochores prevents mitotic checkpoint silencing

The previous data suggest that removal of Mps1 is required for efficient checkpoint silencing, or, alternatively, that persistent kinetochore Mps1 might have caused unstable attachments that engaged the mitotic checkpoint, for instance by increasing local Aurora B activity or otherwise affecting kinetochore function. Five lines of evidence strongly argue against the presence of unstable attachments. First, if Aurora B–mediated destabilization of kinetochore microtubules promoted checkpoint activity in Mis12-Mps1–expressing cells, stabilization of these attachments by inhibition of Aurora B is predicted to revert the extension of metaphase in these cells, much like in the case of monastrol- or taxol-treated cells (Hauf et al., 2003; Yang et al., 2009). However, addition of ZM447439 to cells expressing Mis12-Mps1 did not prevent the mitotic extension (Fig. 3 A). Second, attachment defects that can engage the checkpoint for hours are expected to have at least some effect on ability of cells to quickly biorient all chromosomes (see Hanisch et al., 2006; Gaitanos et al., 2009; Liu et al., 2009; Raaijmakers et al., 2009; and Fig. S2, F and G for examples). Mis12-Mps1–expressing cells did not show any significant increase in prometaphase time (Fig. S2 H). Third, forced anaphase initiation in Mis12-Mps1–expressing, metaphase-arrested cells via addition of Mps1-IN-1 showed no increase in segregation errors compared with control cells (∼10%; Jelluma et al., 2008a,b; Videos 1 and 2; Fig. 3 B). Such increase would be expected if metaphase cells with (minor) attachment defects are induced to undergo anaphase. Fourth, interkinetochore distances of chromosomes in Mis12-Mps1 cells were similar to control cells, with no sisters that were under less tension than any of the sister pairs in control cells (Fig. 3 C). Fifth, overall microtubule density and appearance of cold-stable kinetochore fibers upon Mis12-Mps1 expression was indistinguishable from that of normal cells (Fig. 3 D).

Kinetochore-tethered Mps1 maintains Mad1 and Mad2 on attached, bioriented kinetochores

Despite normal attachment and alignment, Mad1 and Mad2 were localized to kinetochores of bioriented chromosomes in metaphase cells expressing Mis12-Mps1, but not LAP-Mps1 (Fig. 4 A and Fig. S3), and this depended on Mps1 kinase activity (Fig. 4 B). Strikingly, high levels of Mad2, similar to those in nocodazole-treated cells, were apparent on kinetochore pairs that were under full tension (Fig. 4, C and D) and that had normal, cold-stable k-fibers (Fig. 3 D). Importantly, Mis12-Mps1–expressing metaphase cells with kinetochore-bound Mad2 contained Mad2 on all kinetochores (Fig. 4 A). In contrast, metaphase figures of cells with slight destabilization of k-fibers or other attachment defects that allow alignment but significantly prolong prometaphase and metaphase had no or very few kinetochores with detectable Mad1 or Mad2 (Fig. S2 G; Hanisch et al., 2006; Daum et al., 2009; Liu et al., 2009). The results strongly suggest that preventing Mps1 from leaving kinetochores is sufficient to cause persistent mitotic checkpoint-mediated APC/C inhibition, independent of the attachment status of kinetochores, and argue that Mps1 removal is a prerequisite for
Figure 2. Tethering Mps1 to kinetochores extends metaphase. (A) Immunolocalization of LAP-Mis12-Mps1(WT) in U2OS during indicated phases of cell cycle. (B) Percentage of mitotic (Mpm2 positive) U2OS cells that were transfected and treated as indicated, as determined by flow cytometry. Immunoblot shows levels of LAP-Mps1 and LAP-Mis12-Mps1 in total cell lysate. (C and D) Time spent in prometaphase and metaphase of transiently transfected U2OS cells with indicated plasmids and treated as indicated. Each horizontal bar represents a single cell.
mitotic checkpoint silencing. Interestingly, prolonged metaphases with high levels of Mad1 and Mad2 on bioriented chromosomes were also observed when Spindly–Dynein interaction was inhibited but not when Spindly was depleted (Barisic et al., 2010; Gassmann et al., 2010), showing that Spindly removal, like Mps1 removal, is a prerequisite for checkpoint silencing. It may be of interest to examine whether Mps1 influences Spindly function or vice versa.

Figure 3. Sustained Mps1 activity at kinetochores does not affect microtubule attachment or chromosome biorientation. (A) Time spent in prometaphase and metaphase of U2OS cells transfected with indicated plasmids and treated as indicated. Each horizontal bar represents a single cell. (B) Stills of Video 1 showing a LAP-Mis12-Mps1-WT–expressing U2OS cell after Mps1-IN-1 addition during metaphase. Graph shows distribution of time after Mps1-IN-1 addition for cells to initiate anaphase. Average time is 12.4 min ± 3.0 min (SD), n = 45 cells. (C) Distribution of interkinetochore distances in U2OS cells transfected and treated as indicated. For LAP-Mis12-Mps1–expressing cells, only the cells showing clear Mad2 staining on kinetochores were analyzed. (D) Cold-stable microtubules in U2OS cells expressing the indicated constructs. Examples are shown for metaphases that show no, low/medium, or high Mad2 at kinetochores. Averages of absolute total spindle intensities per cell are indicated (±SD).
Figure 4. **Persistent presence of Mps1 maintains Mad1 and Mad2 on attached, bioriented kinetochores.** (A and B) Mad2 localization at kinetochores of U2OS cells transiently transfected with LAP-Mps1-WT or LAP-Mis12-Mps1-WT, treated as indicated. Graph shows distribution of cells with high, low/medium, or no Mad2 signal on kinetochores. (C) Close-up of Mad2 localization at individual kinetochore pairs of U2OS cells treated and transfected as in A. (D) Mad2 intensity related to interkinetochore distance of kinetochore pairs in cells transfected and treated as in A.
A model for mitotic checkpoint regulation by Mps1

Combining the present and recent studies (Howell et al., 2001, 2004; Jelluma et al., 2008a,b; Tighe et al., 2008; Hewitt et al., 2010; Maciejowski et al., 2010; Santaguida et al., 2010; Sliedrecht et al., 2010), we propose the following model for the roles of Mps1 in the control of the metaphase-to-anaphase transition (Fig. 5): in prophase and prometaphase, Mps1 is recruited to unattached and/or tension-less kinetochores where its activity, possibly aided by dimerization, promotes error correction and ensures kinetochore-dependent catalysis of APC/C–Cdc20 inhibitory complexes via establishing kinetochore binding of Mad1, via conformational activation of Mad2, and via inhibition of checkpoint silencing mechanisms. Once activated, Mps1 contributes to its own release from kinetochores to ensure cytoplasmic stabilization of APC/C–Cdc20 inhibitory complexes and to allow its removal from kinetochores for efficient checkpoint silencing once stable, bioriented attachment has been achieved. Deep understanding of how Mps1 promotes these various processes will require the identification of the direct Mps1 substrates and elucidation of the recruitment mechanisms of Mps1 and the checkpoint machinery.

Materials and methods

Cell culture, plasmids, and transfections

U2OS and HeLa cells were grown in DME with 8% FBS, supplemented with penicillin/streptomycin. UTRM10-WT cells (Jelluma et al., 2008a) and UTRM-LAP-Mps1M602G cells (Sliedrecht et al., 2010) were grown in DME with 8% FBS, supplemented with penicillin/streptomycin, and 1 mg ml⁻¹ doxycycline (Sigma-Aldrich) for continuous knockdown of the endogenous protein (Jelluma et al., 2008a). PtK2 cells (a gift from Jagesh Shah, Harvard Institutes of Medicine, Boston, MA) were grown in EMEM with 10% FBS, supplemented with glutamine, nonessential amino acids, and penicillin/streptomycin.

pSuper-Mock, pSuper-Mps1, pcDNA-LAPMps1, and pSuper-Mad2 have been described previously (Kops et al., 2004; Jelluma et al., 2008b). pcDNA-LAP-Mps1-M602G was created by site-directed mutagenesis of pcDNA-LAP-Mps1. Endogenous Mps1 replacement assays were done as in Jelluma et al. (2008b). To add a PTS1 signal to Mps1, point mutations in pcDNA3-LAP-Mps1 construct were introduced by site-directed mutagenesis to alter the last three C-terminal amino acids to Serine-Lysine-Leucine or to Alanine-Lysine-Leucine (Gould et al., 1989). pcDNA-LAP-Mis12-Mps1 constructs were created by inserting the full Mis12 sequence in pcDNA-LAP-Mps1. All sequences were verified by automated sequencing. Plasmid transfections in U2OS cells were done with calcium phosphate, and LAP-Mps1-WT was expressed transiently in PtK2 cells via standard electroporation.

Time-lapse live-cell imaging

U2OS cells were grown in 8-well chambered glass-bottom slides (LabTek), and co-transfected with the indicated plasmids and H2B-pEYFP or H2B-pDSRED for visualization of DNA. Cells were blocked in S phase with 2.5 mM thymidine (Sigma-Aldrich) 24 h after transfection for 24 h. After release from thymidine, mitotic progression was followed with live-cell imaging as described below. For prometaphase time measurements in Fig. S2 F, stable H2B-EYFP-expressing HeLa cells were treated with 10 or 50 µM noscapine (Sigma-Aldrich) or with 20 or 660 nM nocodazole (Sigma-Aldrich). See Vasquez et al. (1997) and Zhou et al. (2002) for effects of noscapine and low nocodazole on microtubule dynamics. Live-cell imaging was done on a from kinetochores after biorientation, checkpoint silencing cannot occur. Question mark indicates the likely contribution of a cycling, and thus also cytoplasmic, pool of Mis12-Mps1 (~60%) with the uncertainty of whether this fusion protein functions as a dimer.
microscope (model IX-81; Olympus), controlled by CellM software (Olympus), in a heated chamber (37°C and 5% CO₂) using a 20x/0.59A UPLFN objective. Every 3 min fluorescent images of H2B-EYFP were acquired (15-msec exposure) with a camera (ORCAER; Hamamatsu Photonics). Images were processed for analysis to maximum intensity projections of all Z-planes and using CellM software.

Immunoblotting and immunofluorescence

Immunoblotting was performed according to standard procedures; antibodies used were anti–Mps1-Nt (Millipore) and anti-α-tubulin (Sigma-Aldrich).

For immunofluorescence analysis, cells were treated 45 min before fixation with the indicated compounds: 660 nM nocodazole (Sigma-Aldrich), 10 μM MI-232 (Sigma-Aldrich), 2 μM 2MA447-459 (Focis Bioscience), 1 μM 23dMB-P1 (a gift from Chao Zhang and Kevan Shokat, UCSF, San Francisco, CA), and 10 μM Mps1-IN-1 (a gift from N. Gray; Kwiatkowski et al., 2010). Cold-shock treatments were done by replacing warm media with ice-cold media for 15 min before fixation. Fixation and immunostaining were done as described previously (Jelluma et al., 2008b). Anti–Mps1-Nt (Millipore), anti–AFP (custom rabbit serum), anti–phospho–Thr232–Aurora B (Rockland), anti–Mad2 (custom rabbit serum), and anti-Mad1 (a gift from Andrea Musacchio, IFOM-IEO, Milan, Italy) were used as primary antibodies and detected with Alexa Fluor 488, 568, or 647 (Invitrogen). Images were acquired at room temperature on a DeltaVision RT system (Applied Precision) with a 100x/1.40 NA UPlanSApo objective (Olympus) equipped with a CoolSnap HQ camera and using SoftWorx software. Images were maximum projections of a deconvolved stack and adjusted (identically within experiments) with SoftWorx and Adobe Photoshop CS3. Quantitations were done as described previously (Jelluma et al., 2008b).

Online supplemental material

Figure S1 shows that Mps1 auto-regulates its turnover at kinetochores. Figure S2 shows that a pool of Mts12-Mps1 is tethered to kinetochores and causes prolonged metaphases without detectable prior defects in prometaphase. Figure S3 shows that Mad1 and Mad2 localize to metaphase kinetochores of Mts12-Mps1–expressing cells. Videos 1 and 2 show two examples of Mts12-Mps1–expressing, metaphase-arrested cells that were forced to initiate anaphase via addition of Mps1-IN-1. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201003038/DC1.

We thank Jagesh Shah and Yinghua Guan for efforts on Mps1 FRAP and for PtK2 cells; Adrian Sauvin, Chao Zhang, Kevan Shokat, Nathanael Gray, Andrea Musacchio, and Suzanne less for providing reagents; the Kops, Medema, and lens laboratories for discussions and insights; and Adrian Sauvin and René Medema for critically reading the manuscript. This work was supported by a VIDI grant of the Netherlands Organization for Scientific Research (NWO-17.776.33.0), an ERC Starting Independent Researcher Grant (KINGDOM) to G.J.P. Kops, and by the Netherlands Genomics Initiative.

Submitted: 9 March 2010
Accepted: 14 September 2010

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Timely anaphase by release of Mps1 from kinetochores

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Online supplemental material
Figure S1. Mps1 auto-regulates its turnover at kinetochores. (A) Western blot showing faster mobility of inactivated Mps1 compared with active Mps1. (B) LAP-Mps1-WT localization at kinetochores of UTRM10-WT cells treated as indicated, detected by immunofluorescence using anti-GFP antibody to detect the LAP-tag. Graph represents quantitation of fluorescence intensities; error bars represent SEM (per condition n = 5 cells, fluorescence intensities of 22 separate kinetochores per cell were determined). Western blot shows total expression levels of uninhibited and inhibited LAP-Mps1-WT. Note that UTRM10-WT cells express near-endogenous levels of LAP-Mps1-WT (Jelluma et al., 2008a), and levels are not changed upon inhibition, as judged by the levels of the loading control α-tubulin. (C) Mps1 localization in UTRM1AP-Mps1M602G cells treated as indicated. Graph shows quantitation of fluorescence intensities at kinetochores; error bars represent SEM (per condition n = 8 cells, fluorescence intensities of 22 separate kinetochores per cell were determined). (D) Phospho-Aurora B intensity levels in UTRM10-WT cells treated as indicated. Graph shows quantitation of fluorescence intensities; error bars represent SD (total cell fluorescence intensities were determined; n = 5 cells [DMSO and ZM447439] or n = 10 cells [Mps1-IN-1]). (E) LAP-Mps1-WT localization at kinetochores of UTRM10-WT cells treated as indicated. (F) Live fluorescence images of U2OS cells transfected with the indicated constructs and treated as indicated. Peroxisomal localization is identifiable by the punctate pattern of fluorescence in the cytosol (Gould et al., 1989). (G) Comparison of FRAP of LAP-Mps1-WT on kinetochores in PtK2 cells treated with nocodazole, MG132, and Mps1-IN-1, and of LAP-Mps1M602G on kinetochores in UTRM1AP-Mps1M602G cells treated with nocodazole, MG132, and 23-dMB-PP1 as measured and shown in Fig. 1 E. (H) Immunolocalization of LAP-Mps1-WT in UTRM10 cells treated as indicated.
A pool of Mis12-Mps1 is tethered to kinetochores and causes prolonged metaphases without detectable prior defects in prometaphase. (A) Localization on kinetochore pairs of the indicated LAP-tagged proteins relative to ACA. Line scans for both signals (LAP and ACA) were made along kinetochore pairs of nocodazole-treated cells and averaged (n = 6 pairs each condition). (B and C) FRAP of LAP-Mis12-Mps1-WT on kinetochores in mitotic U2OS cells. In C, the kinetochore was bleached a second time. (D) Bar graphs showing time spent in prometaphase and metaphase of U2OS cells transiently transfected with indicated plasmids. Each horizontal bar represents a single cell. (E) Graph representing cumulative frequency of anaphase initiation versus time of cells shown in D. Nuclear envelope breakdown is set to t = 0. Western blot shows whole-cell lysate expression levels of indicated plasmids. (F) Box-and-whisker plot showing distributions of prometaphase time of cells treated as indicated. See Materials and methods for use of nocodazole and low doses of nocodazole. (G) Immunolocalization of Mad2 and Hec1 in cells treated with low (metaphase after prolonged prometaphase) or high (prometaphase) doses of nocodazole or nocaspine, as in F. (H) Graph representing cumulative frequency of cells, transfected as indicated, that have reached full alignment.

Figure S2.
Figure S3. **Mad1 and Mad2 localize to metaphase kinetochores of Mis12-Mps1–expressing cells.** Immunolocalization of Mad1 and Mad2 in U2OS cells transfected and treated as indicated.

**Video 1. Mis12-Mps1–expressing, metaphase-arrested cell that was forced to initiate anaphase via addition of Mps1-IN-1, example 1.** A U2OS cell cotransfected with LAP-Mis12-Mps1 and H2B-DSRED was followed by time-lapse microscopy (IX-81 microscope; Olympus). The first frame shows LAP-Mis12-Mps1 expression (EYFP), next frames show DNA (H2B-DSRED), and were taken every 3 min. At 9 min, Mps1-IN-1 was added to force the cell to initiate anaphase.

**Video 2. Mis12-Mps1–expressing, metaphase-arrested cell that was forced to initiate anaphase via addition of Mps1-IN-1, example 2.** A U2OS cell cotransfected with LAP-Mis12-Mps1 and H2B-DSRED was followed by time-lapse microscopy (IX-81 microscope; Olympus). The first frame shows LAP-Mis12-Mps1 expression (EYFP), next frames show DNA (H2B-DSRED), and were taken every 3 min. At 9 min, Mps1-IN-1 was added to force the cell to initiate anaphase.

**References**
