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 (Espiet 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
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-Bluesma 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 complex(es) (Maciejowski et al., 2010).

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-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.

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 (Espet 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.
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(K602G) cells were treated as indicated, and 0.81-µm² 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 bi-oriented chromosomes (Fig. 1 F). Inhibiting Aurora B prevented the accumulation of inhibited Mps1 on non-bi-oriented chromosomes (Fig. S1 H; Santaguida et al., 2010). Thus, recruitment of Mps1 to kinetochores is strongly diminished as soon as chromosomes have bi-oriented. 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-bi-oriented chromosomes where it is rapidly released through a mechanism that involves Mps1-dependent phosphorylation. Upon bi-orientation 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 (Maciejowska et al., 2010). We speculate that Mps1 dimerization (Hewitt et al., 2010) and/or targeting via the Mps1-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 monastral- 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 than that of 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, bi-oriented kinetochores

Despite normal attachment and alignment, Mad1 and Mad2 were localized to kinetochores of bi-oriented 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-Mps12-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-Mps1Δ C20 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). PK2 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 PK2 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...
microscope (model IX-81; Olympus), controlled by CellM software (Olympus), in a heated chamber (37°C and 5% CO2) using a 20x/0.5NA UPLFL 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).

**Flow cytometry analysis**

Cells were released from a 24-h thymidine-induced block into nocodazole for 16 h. The double exponential that we observe consists of a fast component in the 25 × 25-pixel square during the entire measurement. FRAP analysis: Log transformation of the FRAP data indicates that in our experiments the recovery follows a double exponential (log transformation was done by plotting $-\ln([F(t) - F(0)] / [F(0) - F])$ vs. time where $F$ is the fluorescence reached at the plateau, $F(0)$ is the fluorescence at a certain time point, and $F(t)$ is the fluorescence observed immediately after bleaching, as was done in Howell et al. (2004). The double exponential that we observe consists of a fast component that equals the kinetics found for cytoplasmic LAP-Mps1 $^{M60G}$, both in the uninhibited and inhibited states. The slow component in the double exponential represents the LAP-Mps1 kinetics on the kinetochore. Recovery half-times were determined by double-exponential curve fitting using GraphPad Prism software that confirmed the log transformation analysis. FRAP measurements of LAP-Mps1 in PtK2 cells, after inhibition with 10 µM Mps1-IN-1, were done as described above.

**Flow cytometry analysis**

Cells were released from a 24-h thymidine-induced block into nocodazole for 16 h and analyzed by flow cytometry as described previously (Kops et al., 2005). Flow cytometric analysis of transfected cells was based on Spectrin-GFP expression.

**Measurement of sister interkinetochore distances**

Cells were grown on cover-glasses, transfected, treated, and fixed as described above. Center-to-center distances of the ACA dots of sisters within the same focal plane were measured using SoftWorx software.

**Online supplemental material**

Figure S1 shows that Mps1 auto-regulates its turnover at kinetochores. Figure S2 shows that a pool of Msi12-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 Msi12-Mps1–expressing cells. Videos 1 and 2 show two examples of Msi12-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.

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**References**


Hardwick, K.G., E. Weiss, F.C. Luca, M. Winne, and A.W. Murray. 1996. Activation of the budding yeast spindle assembly checkpoint without...


