Spindle assembly checkpoint robustness requires Tpr-mediated regulation of Mad1/Mad2 proteostasis

The MIT Faculty has made this article openly available. Please share how this access benefits you. Your story matters.

<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>As Published</td>
<td><a href="http://dx.doi.org/10.1083/jcb.201309076">http://dx.doi.org/10.1083/jcb.201309076</a></td>
</tr>
<tr>
<td>Publisher</td>
<td>Rockefeller University Press</td>
</tr>
<tr>
<td>Version</td>
<td>Final published version</td>
</tr>
<tr>
<td>Accessed</td>
<td>Mon Apr 25 05:18:54 EDT 2016</td>
</tr>
<tr>
<td>Citable Link</td>
<td><a href="http://hdl.handle.net/1721.1/89126">http://hdl.handle.net/1721.1/89126</a></td>
</tr>
<tr>
<td>Terms of Use</td>
<td>Creative Commons Attribution-Noncommercial-Share Alike</td>
</tr>
<tr>
<td>Detailed Terms</td>
<td><a href="http://creativecommons.org/licenses/by-nc-sa/3.0/">http://creativecommons.org/licenses/by-nc-sa/3.0/</a></td>
</tr>
</tbody>
</table>
Spindle assembly checkpoint robustness requires Tpr-mediated regulation of Mad1/Mad2 proteostasis

Nina Schweizer,1 Cristina Ferrás,1 David M. Kern,3,4 Elsa Logarinho,1,2 Iain M. Cheeseman,3,4 and Helder Maiato1,2
1Chromosome Instability and Dynamics Laboratory, Instituto de Biologia Molecular e Celular, and 2Cell Division Unit, Department of Experimental Biology, Faculdade de Medicina, Universidade do Porto, 4099-002 Porto, Portugal
3Whitehead Institute for Biomedical Research and 4Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02142

Introduction

The spindle assembly checkpoint (SAC) ensures correct chromosome segregation by providing time for proper kinetochore (KT) attachment to spindle microtubules (MTs) through inhibition of the anaphase-promoting complex (APC; Musacchio and Salmon, 2007). Critical to this inhibition is the repression of the APC activator Cdc20 by Mad2, thereby preventing premature degradation of cyclin B and securin. Mad2 exists in two distinct pools at KTs: one that is stable and another with high turnover (Shah et al., 2004; Vink et al., 2006). The stable pool of Mad2 is bound to Mad1, adopting a structural conformation known as closed-Mad2 (c-Mad2; Sironi et al., 2002; Luo et al., 2004; De Antoni et al., 2005; Mapelli et al., 2007). The Mad1–c-Mad2 complex at unattached KTs acts as a receptor for an inactive cytosolic open-Mad2 (o-Mad2) conformer that is converted into active c-Mad2 by binding to this template. c-Mad2 is selectively incorporated into the mitotic checkpoint complex (MCC), which is composed of Cdc20, BubR1, and Bub3 and inhibits the APC (Sudakin et al., 2001; Sironi et al., 2002; Luo et al., 2004; Mapelli et al., 2007; Tipton et al., 2011; Chao et al., 2012).

In addition to their localization to KTs, Mad1 and Mad2 are also recruited to the nuclear pore complex (NPC) by the inner nuclear pore protein Tpr, which has been shown to be required for normal SAC response from yeast to humans (Campbell et al., 2001; Ikui et al., 2002; Iouk et al., 2002; Scott et al., 2005; Lee et al., 2008; De Souza et al., 2009; Lince-Faria et al., 2009; Ding et al., 2012). However, the underlying molecular mechanism remains unclear. Here, we dissect how human Tpr regulates the SAC response and propose a mechanism by which Tpr association with Mad1 and Mad2 ensures proper SAC proteostasis throughout the cell cycle that is required to mount and sustain a robust SAC response.
Results and discussion

Tpr is required to sustain a robust SAC response

To determine whether Tpr contributes to SAC robustness, we analyzed mitotic duration using live-cell imaging in control and Tpr-depleted HeLa cells after RNAi, with and without nocodazole (Fig. 1, A–C). Control cells progressed from nuclear envelope breakdown (NEB) to anaphase in 24 ± 5 min, whereas Tpr-depleted cells took 22 ± 5 min (median ± SD, n = 100 cells/condition; Fig. 1 B). This difference is statistically significant (P < 0.01), especially in the presence of nocodazole (control = 16.5 ± 7.6 h, Tpr RNAi 11.7 ± 7.1 h; median ± SD, n = 350 cells/condition, P < 0.001; Fig. 1, A and C). Most cells in either experimental condition died after this prolonged mitotic arrest, but cell death occurs significantly earlier in Tpr-depleted cells (control, 15.0 ± 7.0 h; Tpr RNAi, 11.4 ± 6.9 h; median ± SD, n = 320 cells/condition, P < 0.001; Fig. 1, D and F). A minor fraction of cells undergo mitotic slippage, which also occurs significantly earlier in Tpr-depleted cells (control, 29.2 ± 6.2 h; Tpr RNAi, 13.3 ± 8.8 h; median ± SD, n = 30 cells/condition, P < 0.001; Fig. 1, E and F). Together, ~40% of Tpr-depleted cells exit mitosis during the first 10 h of nocodazole treatment, a twofold increase relative to controls (Fig. 1 G).

To confirm the specificity of the phenotype, we performed a rescue experiment using HeLa cells stably expressing an RNAi-resistant mouse Tpr fused to GFP (Fig. 1 H), which interacts with human Mad1 and Mad2 (Hutchins et al., 2010). Tpr-GFP-expressing cells depleted of endogenous Tpr spent equivalent times in mitosis after nocodazole treatment when compared with control HeLa cells (19 ± 5.7 h vs. 16.5 ± 7.6 h; median ± SD, n = 103 cells, P = 0.07; Fig. 1 I), demonstrating the specificity of the Tpr depletion phenotypes. Thus, Tpr is required for a robust SAC response, supporting previous claims for the existence of intermediate response regimens (Lince-Faria et al., 2009) that cannot be explained by “all-or-nothing” SAC models.

Tpr is required for the localization of a fraction of c-Mad2, but not Mad1, to unattached KTs

We have been unable to detect endogenous or GFP-tagged Tpr at KTs by fluorescence microscopy (Lince-Faria et al., 2009; and unpublished data). Previous studies also reported conflicting findings regarding the effect of Tpr depletion on the KT localization of the SAC proteins Mad1 and Mad2 (Lee et al., 2008; Lince-Faria et al., 2009). To clarify this, we performed quantitative immunofluorescence microscopy with specific antibodies against Cdc20, BubR1, Mad1, and total Mad2 (t-Mad2), as well as the c-Mad2 (Fava et al., 2011) and o-Mad2 (Hewitt et al., 2010) conformers, in nocodazole-treated control and Tpr-depleted cells (Fig. 2 A). After Tpr RNAi, t-Mad2 levels at KTs decrease by ~50%, with equivalent reductions in the levels of c-Mad2 and o-Mad2 (Fig. 2 B). In contrast, Mad1 levels at KTs increase by ~40%, whereas Cdc20 and BubR1 increase by ~80% (Fig. 2 B), which might reflect a feedback response caused by an effective reduction of cytosolic MCC. This would be consistent with previous work in yeast that showed that alteration of the normal Mad1/Mad2 ratio compromises SAC response (Barnhart et al., 2011). Importantly, c-Mad2 levels at unattached KTs are also reduced after Tpr depletion using a distinct siRNA target sequence (Rajanala and Nandicoori, 2012) and are restored by expression of RNAi-resistant mouse Tpr-GFP (Figs. 2 B and S1, A–D), ruling out possible Mad2 off-targeting effects. Altogether, these results indicate that normal localization of c-Mad2 and o-Mad2 to unattached KTs is compromised after Tpr depletion.

The reduction of o-Mad2 at KTs in Tpr-depleted cells might reflect a proportional reduction of its KT receptor, c-Mad2, or altered turnover at KTs. To distinguish between these possibilities, we performed FRAP analysis of GFP-Mad2 at KTs in control and Tpr-depleted cells treated with nocodazole. In both conditions, GFP-Mad2 displays indistinguishable, rapid recovery with single exponential kinetics (control, t½ = 6 ± 8 s, n = 10 KTs; Tpr RNAi, t½ = 4 ± 3 s, n = 8 KTs; median ± SD, P = 0.22) with a similar extent of recovery (control, 58 ± 18%, n = 10 KTs; Tpr RNAi, 57 ± 14%, n = 8 KTs; median ± SD, P = 0.22; Fig. 2 C). These findings demonstrate that Tpr does not impact Mad2 dynamics at unattached KTs, which suggests that Tpr promotes the localization of c-Mad2 to unattached KTs and is rate-limiting for the conversion of o-Mad2 into cytoplasmic c-Mad2.

Tpr interacts with Mad1 and Mad2 throughout the cell cycle

Tpr interacts with Mad1 and Mad2 (Lee et al., 2008; Lince-Faria et al., 2009). To investigate the nature of these interactions, we isolated GFP-Mad1 from nocodazole-arrested mitotic cells using a cross-linking strategy and mass spectrometry. In parallel, we performed coimmunoprecipitation (co-IP) experiments with endogenous Mad1 in asynchronous or mitotic enriched populations (Fig. 3 B). We found that Mad1 associates with Tpr, cyclin B/Cdk1, and Mad2, but failed to detect Cdc20, BubR1, or Bub3 (Fig. 3, A and B), which suggests that the TM2 complex exists independently of the MCC throughout the cell cycle. Importantly, IP of a GFP-Mad1 mutant (KVLHM-5A) that is unable to interact with Mad2 (Kim et al., 2012) also isolated Tpr, which suggests that Tpr interaction with Mad1 is independent of Mad2 (Fig. 3 A). To test where these proteins interact, we performed an in situ proximity ligation assay (PLA; Söderberg et al., 2006). This revealed abundant fluorescent PLA foci between Tpr and Mad1 at the nuclear periphery, likely corresponding to NPCs (Fig. 3, C and D). Tpr depletion by RNAi or a PLA reaction between the core nuclear pore protein Nup107 and Mad1 abolished the formation of fluorescent foci (Fig. 3, C and D). No Tpr/Mad1 PLA foci were ever detected at unattached KTs in cells treated with nocodazole (Fig. 3 C). These findings, together with our IP experiments, strongly suggest that Tpr interacts with Mad1 at the nuclear envelope during interphase, but not at KTs during mitosis. Consistent with this conclusion, Tpr depletion disrupted the localization of both Mad1 and c-Mad2 at NPCs during interphase (Fig. 3 E).
Tpr is rate-limiting for the amount of Mad1–c-Mad2 produced before mitosis
The previous data raised the possibility that Tpr may function as a scaffold for regulating the assembly or stability of Mad1–c-Mad2 before their targeting to KTs. To test this hypothesis, we performed IP experiments to investigate the amount of Mad1–c-Mad2 produced in asynchronous and mitotic extracts. We found that Tpr depletion results in ~50%...
Tpr is required for c-Mad2, but not Mad1, localization at unattached KTs. (A) Immunofluorescence of nocodazole-treated control and Tpr-depleted HeLa cells with the indicated antibodies. Bar, 5 µm. (B) Normalized fluorescence signals at KTs from Tpr-depleted cells relative to controls (set to 1). Error bars represent standard deviations from the mean obtained from two or three independent experiments. Green and red boxes correspond to parental HeLa cells.
**Table A**

<table>
<thead>
<tr>
<th>IP</th>
<th>% Seq Coverage</th>
<th># Peptides</th>
<th>MW (kD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mad1</td>
<td>71</td>
<td>1127</td>
<td>83.1</td>
</tr>
<tr>
<td>Mad2</td>
<td>10.2</td>
<td>3</td>
<td>23.5</td>
</tr>
<tr>
<td>Tpr</td>
<td>6.9</td>
<td>18</td>
<td>267.3</td>
</tr>
<tr>
<td>Cyclin B</td>
<td>5.8</td>
<td>4</td>
<td>48.3</td>
</tr>
<tr>
<td>Cdk1</td>
<td>29</td>
<td>13</td>
<td>34.1</td>
</tr>
</tbody>
</table>

**Table B**

<table>
<thead>
<tr>
<th></th>
<th>Async</th>
<th>Mitosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>IP</td>
<td>NB</td>
<td>PC</td>
</tr>
<tr>
<td>Tpr</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mad1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>t-Mad2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Figure 3. Tpr interacts with Mad1 and Mad2 throughout the cell cycle.** (A) LAP-Mad1 purification followed by mass spectrometry analysis reveals interactions with Mad2, Cyclin B, Cdk1, and Tpr during mitosis. A Mad1 mutant (KVLHM-5A) that does not bind Mad2 is still able to interact with Tpr. (B) IP from asynchronous (Async) and mitotic HeLa cells (Mitosis) using a Mad1 antibody. I, input; PC, pre clear; IP, immunoprecipitated; NB, nonbinding fraction. All fractions were analyzed by WB for detection of Tpr, Mad1, and t-Mad2. (C) 3D image projections from PLA experiments between Tpr and Mad1 at NPCs in interphase and unattached KTs in mitotic HeLa cells treated with nocodazole. Green foci indicate a positive reaction, and nuclei/chromosomes were counterstained with DAPI. PLA between Nup107 and Mad1, as well as between Tpr and Mad1 upon Tpr RNAi were used as negative controls. Bars, 5 µm. (D) Quantification of the PLA foci at the nuclear envelope (NE) for Tpr/Mad1, Nup107/Mad1, and Tpr/Mad1 upon Tpr RNAi. Error bars represent standard deviations from three independent experiments. (E) Immunofluorescence with Tpr, Mad1, and c-Mad2 antibodies. Tpr depletion prevented Mad1 and c-Mad2 localization at the nuclear envelope. + and − signs indicate nondepleted and depleted cells, respectively. Bar, 10 µm.

Reduction of Mad1 and Mad2 that co-IP in both asynchronous and mitotic populations (Fig. 4, A and B). Importantly, we found that the starting amount of Mad1 and Mad2 present in the extracts (input) proportionally decreases after Tpr depletion (Fig. 4, A and B), which suggests that Tpr affects the cellular pool of Mad1 and Mad2.

Gray boxes correspond to HeLa cells stably expressing Tpr-GFP or GFP-Mad2. (C) FRAP of GFP-Mad2 in control and Tpr-depleted HeLa cells. Data points represent means obtained from 10 (control) or 8 (Tpr RNAi) KTs, to which a single exponential curve was fit. Error bars represent standard deviation.
To test whether Tpr is required for the expression or stability of SAC-related proteins, we performed a semiquantitative Western blot (WB) analysis using asynchronous and mitotic extracts derived from control and Tpr-depleted cells. We found that the protein levels of Mad1, Mad2, and Mps1, but not Cdc20, decrease after Tpr depletion (Fig. 4 C). Moreover, the observed decrease was proportional to the extent of Tpr depletion (Fig. 4 D). Overall, these data suggest that the interaction between Tpr, Mad1, Mad2, and Mps1 is rate-limiting for the amount of Mad1-c-Mad2 produced before mitosis. In support of this hypothesis, we found that even partial knockdown of Mad1, Mad2, or Mps1 in asynchronous cells decreases the available pool of all the other SAC proteins interacting with Tpr, including Tpr itself (Fig. 4 E).

Tpr associates with Mad1-c-Mad2 independently of KTs and Mps1 kinase activity

Mps1 kinase activity is essential to recruit Mad1-c-Mad2 to unattached KTs (Maciejowski et al., 2010; Santaguida et al., 2010; Sliedrecht et al., 2010; Fig. S2, A–C). Thus, the decrease in Mad2 levels at unattached KTs observed after Tpr RNAi may be a consequence of reduced Mps1 levels or activity. To test these possibilities, we started by partially depleting Mps1...
(Fig. S1 E), and quantified Mad1 and c-Mad2 levels at unattached KTs by fluorescence microscopy. In contrast to the decrease observed in c-Mad2, but not Mad1, after Tpr depletion, we found that both Mad1 and c-Mad2 levels are reduced to a similar extent after partial Mps1 depletion (Fig. S1, F–H). This suggests that the role of Tpr in regulating c-Mad2, but not Mad1, levels at KTs is independent of Mps1. Finally, we determined whether Mps1 kinase activity is required for the interaction between Mad1, Mad2, and Tpr. Based on Tpr IP, Tpr still interacts with Mad1 and Mad2 in mitotic extracts after Mps1 inhibition (Fig. 4 F). Thus, TM2 complex formation is independent of KTs and Mps1 kinase activity.

Tpr is required for SAC proteostasis throughout the cell cycle

It has been proposed that Tpr acts at NPCs as part of a surveillance system that regulates the export of unspliced mRNA into the cytoplasm (Green et al., 2003; Galy et al., 2004; Vinciguerra et al., 2005; Xu et al., 2007; Coyle et al., 2011; Rajanala and Nandicoori, 2012). We tested whether the observed deficit in SAC protein levels reflects a defect in mRNA transcription/processing by quantifying the content of mature mRNA of several SAC genes with real-time PCR. We found similar expression levels for mad2, mps1, p31, and cdc20 mRNAs in control and Tpr-depleted cells, either in interphase or mitosis (Fig. 5, A and B). Interestingly, mad1 mRNA expression in interphase is significantly increased (P < 0.01) after Tpr depletion (Fig. 5 A), which might reflect a feedback response caused by reduced Mad1 protein levels. Overall, these results demonstrate that the decrease in Mad1, Mad2, and Mps1 after Tpr depletion is not caused by a reduction of the respective mRNAs.

Because Tpr interacts with Mad1, Mad2, and Mps1 and the overall levels of these proteins were reduced upon Tpr depletion, we next investigated a possible role of Tpr in SAC proteostasis by comparing Mad1, Mad2, and Mps1 protein levels in Tpr-depleted extracts derived from asynchronous, G2-enriched, and mitotic cells. In addition, we evaluated whether inhibition of the 26S proteasome by addition of MG132 rescues the effect of Tpr depletion. We found that only asynchronously growing cells partially recover Mad1, Mad2, and Mps1 protein levels after a 2-h treatment with MG132 (Fig. 5 C), which suggests that Tpr regulates the degradation of these proteins before G2 and the commitment to mitosis. Next, we tested whether the observed Mad1, Mad2, and Mps1 degradation after Tpr depletion is responsible for the reduced production of Mad1–c-Mad2 by c-Mad2 IP from control and Tpr-depleted interphase cells in the presence or absence of MG132. We found that Tpr depletion causes a decrease in Mad1 and c-Mad2 present in the extract (input), as well as in the Mad1–c-Mad2 levels. This decrease was partially rescued by incubation with MG132 for 2 h (Fig. 5 D). Finally, we tested whether Tpr is required for the normal turnover of Mad1, Mad2, or Mps1 by treating control and Tpr-depleted cells with cycloheximide to inhibit de novo protein synthesis and monitored the respective protein half-life by WB. We found that Tpr-depleted cells show a significantly shorter half-life for Mad1, Mad2, and, to a lesser extent, Mps1 (Fig. 5, E and F). Based on these experiments, we conclude that Tpr limits the production of Mad1–c-Mad2 by regulating SAC proteostasis.

Overexpression of Mad2 restores Mad2 KT levels and SAC robustness in Tpr-depleted cells

Next, we tested whether the reduced Mad2 protein levels are responsible for the weaker SAC response in Tpr-depleted cells by investigating the ability of Mad2 overexpression to rescue Tpr depletion. For this purpose, we used a cell line expressing ~1.5x GFP-Mad2 levels relative to endogenous Mad2 (Fig. S1 I). First, we quantified Mad2 levels at unattached KTs in control and Tpr-depleted cells overexpressing GFP-Mad2 by immunofluorescence microscopy upon MT depolymerization with nocodazole (Figs. 2 B and S1 J). We found that GFP-Mad2 overexpression restores t-Mad2 levels at unattached KTs to ~90% of control levels in Tpr-depleted cells (Fig. 2 B). Next, we quantified the mitotic duration after nocodazole treatment in Tpr-depleted cells overexpressing GFP-Mad2 and found a significant rescue of mitotic duration relative to parental cells depleted of Tpr (15.8 ± 7.6 h vs. 11.7 ± 7.1 h; n = 115 cells, P < 0.01; Fig. 1 I). We conclude that the proteolytic degradation of Mad2 caused by Tpr depletion is the leading cause of reduced Mad2 levels at unattached KTs and, consequently, of the weaker SAC response.

A mechanism for regulation of SAC proteostasis and robustness by Tpr

Purified Mad1 forms a high-affinity and stable complex with Mad2 in vitro (Sironi et al., 2002; De Antoni et al., 2005), and Mad1 interacts with Mad2 throughout the cell cycle (Chen et al., 1999; Chung and Chen, 2002; Fava et al., 2011). Here we propose that the TM2 complex stabilizes Mad1–c-Mad2 in vivo, preventing it from being degraded by the proteasome. Interestingly, Tpr and respective orthologues in yeast and plants have been implicated in protein SUMOylation at the nuclear periphery, possibly by locally regulating the targeting and/or function of the SUMO-isopeptidases Ulp1/SENP1/SENP2 (Zhao et al., 2004; Xu et al., 2007; David-Watine, 2011). Here we found that the TM2 complex stabilizes Mad1–c-Mad2 in vivo, preventing it from being degraded by the proteasome. Interestingly, Tpr depletion also affects Mad1–c-Mad2 recruitment to NPCs, Mad1–c-Mad2 is not able to interact with either SENP1 or SENP2 at the NPCs, which might alter normal Mad1–c-Mad2 proteostasis. In agreement with this model, either SENP1 or SENP2 depletion by RNAi reduces total Mad1 and Mad2 levels by ~50% (Fig. 5 H). Given so, it is tempting to speculate that the requirement of Tpr for a robust SAC response by regulating Mad1 and Mad2 protein levels before mitosis might involve spatial control of SUMO-mediated proteolysis at the NPCs, but further studies will be necessary to directly test this hypothesis.

Materials and methods

Cell culture and drug treatments

HeLa or U2OS cells were cultured in DMEM with 10% FBS (Invitrogen) in a 37°C incubator with 5% CO2 and a humidified atmosphere. MT
Figure 5. Tpr is required for SAC proteostasis throughout the cell cycle. (A and B) Normalized expression of tpr, mad1, mad2, mps1, cdc20, and p31 in control and Tpr-depleted asynchronous or mitotic cells. Error bars represent standard deviations from three independent experiments. (C) WB analysis of asynchronous, G2, and mitotic enriched HeLa cell extracts from control (+) and Tpr-depleted (−) cells with (+) or without (−) MG132 with the indicated antibodies. The percentage of protein levels relative to controls is indicated. (D) c-Mad2 IP from asynchronous and mitotic HeLa cells with (+) or without (−) Tpr, in the presence (+) or absence (−) of MG132. (E and F) Cells with or without Tpr were treated with cycloheximide (CHX) for various time points as indicated. Total protein extracts of asynchronous HeLa cells were analyzed by WB to detect Tpr, Mps1, Mad1, and t-Mad2 in control and Tpr-depleted cells. α-Tubulin was used as a loading control. *, P < 0.05; **, P < 0.01; ***, P < 0.001. The error bars indicate standard deviations from two independent experiments. (G) Immunodetection of Mad1 (red), SENP1, and SENP2 (green) in Tpr-depleted cells (−). A nondepleted cell (+) was used as internal control. DNA was counterstained with DAPI (blue). (H) WB analysis of asynchronous cell extracts in control and after SENP1 or SENP2 RNAi. The percentage of protein levels relative to controls is indicated. α-Tubulin was used as a loading control. Bar, 10 µm.
Cells were grown on poly-lysine–coated coverslips and fixed either with 4% paraformaldehyde or ice-cold methanol, then extracted subsequently or simultaneously with 0.1–0.2% Triton X-100. After short washes in PBS and blocking with 10% FBS in PBS, cells were incubated with primary antibodies after short washes and incubation with the respective secondary antibodies. DNA was counterstained with DAPI (1 µg/ml; Sigma-Aldrich) before coverslips were mounted in 90% glycerol + 0.3% Trc, pH 8.5, + 0.5% Npropyl gallate on glass slides. Images were acquired on a AxioImager Z1 (63× Plan-Apochromatic oil differential interference contrast objective lens, 1.4 NA) equipped with a CCD (Axiocam MR) camera using the Zen software (all from Carl Zeiss) and blind deconvolved using AutoQuant X (Media Cybernetics). Images were processed in Photoshop CS4 (Adobe) and represent either maximum projections of a deconvolved z stack or a single slice in the case of Figs. 3 E and 3 G. Mouse anti-Mad1 (generated against full-length Mad1, 1:500; provided by P. Meraldi, University of Geneva, Switzerland), mouse anti-c-Mad2 (generated against full-length Mad2, 1:500; provided by A. Samantara and E. Nigg, Biozentrum, Basel, Switzerland), rabbit anti-Mad1 (generated against full-length Mad1, 1:1,000; provided by P. Meraldi, University of Geneva, Switzerland), mouse anti-c-Mad2 (generated against full-length Mad2, 1:500; provided by A. Samantara, rabbit anti-mad2 (1:300; Bethyl Laboratories, Inc.), sheep anti-o-Mad2 (generated against full-length Mad2, 1:200; provided by S. Taylor, University of Manchester, UK), rabbit anti-Cdc20 (1:100; Santa Cruz Biotechnology, Inc.), mouse anti-Mps1 antibody (NT clone 3-742-1, 1:500; Merck Millipore), rabbit anti-Mad2 antibody (A300-301 A-2, 1:1,000; Bethyl Laboratories, Inc.), rabbit anti-Mad1 antibody (1:1,000; provided by P. Meraldi), mouse anti-Mad1 (1:100; Santa Cruz Biotechnology, Inc.), rabbit anti-Cdc20 antibody (sc-2926, 1:1,000; Santa Cruz Biotechnology, Inc.), rabbit anti-SENP1 or -SENP2 (1:2,000), mouse anti-GFP (1:1,000; Invitrogen), and mouse anti-v-tubulin antibody (clone B-512, 1:1,500; Sigma-Aldrich). Goat anti-rabbit, anti-mouse, or anti–sheep antibodies were used as secondary antibodies (1:5,000; Jackson Immunoresearch Laboratories, Inc.). The WBs were quantified using a calibrated densitometer (GS800; Bio-Rad Laboratories) and Quantity One 1-D Analysis Software, version 4.6 (Bio-Rad Laboratories), which highlights saturated pixels when signal intensity falls off the linear range. Only exposures without saturated pixels were used and were normalized for the loading control (α-tubulin).

Local affinity purification (LAP)-Mad1 purification and mass spectrometry

GFP-mad1 was generated in HeLa cells as described previously (Cheeseman et al., 2004). In brief, a pBABE (MMTV)-based plasmid containing GFP-mad1 (or mutant) was cotransfected into 293-GFP cells and clones containing GFP- and DNA containing virus. After infection with the retrovirus, HeLa cells were selected using 2 µg/ml Blasticidin. Resistant cells were sorted by FACS to generate clonal cell lines. To conduct the affinity purifications, cell lines were grown to 70–90% confluency, and then treated with 3.33 µm nocodazole for 16 h. A mitotic shake-off was then performed and cells were pelleted at 1,000 g. A modified cross-linking protocol was developed based on Klokkenbuch and Kast (2010). In brief, the cell pellets were resuspended with 0.4–1% formaldehyde in PBS and rocked gently for 10 min. After pelleting at 1,000 g, the cell pellet was quenched with 0.125 M glycine in PBS for 10 min. The cells were processed by sonication and treatment with detergent to solubilize cross-linked cell material, then a one-step LAP purification was performed as described previously (Cheeseman and Desai, 2003) in the presence of 300 mM KC1. In brief, processed lysate was passed to anti-GFP coupled beads for 1 h. The beads were then washed, and bound protein was eluted with 0.1 M glycine. Elutions were TCA-precipitated, and the protein pellet was resuspended with 8 M urea. Cross-linking reversal was performed at 95°C for 5 min before tryptic digestion. Purified proteins were identified by mass spectrometry using an ion trap mass spectrometer (LTQ XL; Thermofisher Scientific) with a reverse phase gradient over C18 resin (Phenomenex) and SEQUEST software as described previously (Washburn et al., 2001).

PLA

For PLA, we used primary antibodies raised in different species against Tpr (Novus Biologicals) or Nup107 (provided by V. Doye, Institute Jacques Monod, Paris, France) and Mad1 (provided by A. Samantara), which were sequentially detected by species-specific secondary antibodies conjugated with PLA probes according to the manufacturer’s instructions (Duolink, Olink Bioscience).

RNA extraction and transcription analysis by RT-qPCR

Total RNA was isolated from interphase, and mitotic HeLa cells (shake-off) were treated with nocodazole and MG132 using an RNeasy Mini kit according to the manufacturer’s instructions (Qiagen). cDNA synthesis, 1 µg of total RNA was transcribed with the iScript Select cDNA Synthesis kit (Bio-Rad Laboratories) using the random primers and oligo(dT)20 supplied, following the manufacturer’s instructions. For each analysis, gapdh was used for normalization. RT-PCR experiments were performed in the iCycler IQ5 Real-Time PCR Detection System (Bio-Rad Laboratories). The data obtained were analyzed using the Bio-Rad IQ5 Optical System Software v2.1 (Bio-Rad Laboratories).
Transgenic cell lines
A cell line stably expressing GFP-Mad2 was established by transfecting HeLa cells with a pEGFP-C1-Mad2 plasmid (provided by P. Meraldi) using FuGene HD (Roche). The Tpr-GFP cell line was provided by MitoCheck. The Msi1Δ-Mps1 U2OS cell line was provided by G. Kops (University Medical Center, Utrecht, Netherlands).

RNAs
Depletion of Tpr, Mps1, Mad1, Mad2, SENP1, and SENP2 was performed by transfecting HeLa cells with siRNA oligonucleotides directed against the following target sequences: Tpr, 5′-GGUGGAGCAGCAACAAGC-3′ or 5′-GCACACAGGAAAUGUGUUA-3′; Mps1, 5′-GGUGAGUUUGGUUGUCGCAA-3′; Mad1, 5′-CCAAGUGGCGACAGGAG-3′; Mad2, 5′-CCGGUGGUAUAUCCGUCU-3′; SENP1, 5′-UUCUCCACCUCCUGCAUGUUC-3′; and SENP2, 5′-UAUUGUUGGUUAUUGGGAUU-3′ (all from Sigma-Aldrich), using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer’s instructions.

Statistical analysis
Statistical analysis was performed using either Graphpad Prism version 5, OriginPro 8 (Originlab), or SPSS (SPSS advanced statistics 17.0). P-values were calculated with either a Student’s t test or analysis of variance (ANOVA).

Online supplemental material
Fig. S1 shows experiments in support of the specificity of the Tpr phenotype. Fig. S2 shows the effect of Mps1 inhibition for the loading and maintenance of Mad1-c-Mad2 at unattached KTs. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201309076/DC1.

We would like to thank Filipe Pinto, Martina Barisic, and Marin Barisic for technical help, and Carolina Lemos for help with statistical analysis. We also thank all colleagues who kindly provided reagents used in this study.

Author contributions: N. Schweizer and C. Ferrás performed most of the experiments with contributions from I.M. Cheeseman and D.M. Kern. E. Logarinho provided the GFP-Mad2 cell line. N. Schweizer, C. Ferrás, and H. Maiato analyzed the data and wrote the manuscript. H. Maiato designed experiments and coordinated the project.

Submitted: 16 September 2013
Accepted: 11 November 2013

References

Published December 16, 2013

Mapelli, M., L. Massimiliano, S. Santaguida, and A. Musacchio. 2007. The Mad2 conformational dimer: structure and implications for the spindle


