Probing the *in vivo* function of Mad1:C-Mad2 in the spindle assembly checkpoint

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The spindle assembly checkpoint (SAC) restrains anaphase until all chromosomes become bi-oriented on the mitotic spindle. The SAC protein Mad2 can fold into two distinct conformers, open (O) and closed (C), and can asymmetrically dimerize. Here, we describe a monoclonal antibody that specifically recognizes the dimerization interface of C-Mad2. This antibody revealed several conformation-specific features of Mad2 in human cells. Notably, we show that Mad2 requires association with Mad1 to adopt the closed conformation and that the activity of the Mad1:C-Mad2 complex undergoes regulation by p31comet-dependent ‘capping’. Furthermore, C-Mad2 antibody microinjection caused an abrupt termination of the SAC and accelerated mitotic progression. Remarkably, microinjection of a Mad1-neutralizing antibody triggered a comparable mitotic acceleration. Our study provides direct *in vivo* evidence for the model that a kinetochore complex of Mad1:C-Mad2 acts as a template to sustain the SAC and it challenges the distinction between SAC and mitotic timer.

The EMBO Journal (2011) 30, 3322–3336. doi:10.1038/emboj.2011.239; Published online 19 July 2011

Subject Categories: cell cycle

Keywords: closed-Mad2; Mad2 template model; mitotic timing; p31comet; spindle assembly checkpoint

Introduction

Eukaryotic cells have evolved a surveillance mechanism, called spindle assembly checkpoint (SAC), that ensures faithful segregation of the replicated genome during mitosis. This pathway is devoted to restraining anaphase onset until chromosome bi-orientation has occurred. The molecular components of the SAC are conserved among eukaryotes and include Mad1, Mad2, Bub1, Bub3, BubR1/Mad3 and

Mps1 (see Musacchio and Salmon, 2007 for review). The target of the SAC is Cdc20, a cofactor of the anaphase promoting complex/cyclosome (APC/C), that functions as a ubiquitin ligase for degradation of Cyclin B1 and Securin (Peters, 2006). During mitosis, both checkpoint proteins and Cdc20 associate with kinetochores (KTs), where Mad2, BubR1 and Bub3 display high turnover (Howell *et al*, 2004). The same checkpoint proteins can also be found to physically associate with Cdc20 in the cytosol (Sudakin *et al*, 2001). This and other evidence (Rieder *et al*, 1995) led to the model that unattached KT act as catalytic platforms to generate the SAC signal (Musacchio and Salmon, 2007).

The mechanistic aspects of the KT-dependent catalytic generation of an anaphase inhibitor remain to be understood. Biochemical approaches indicate that both Mad2 and BubR1 directly interact with Cdc20 and can inhibit APC/C^{Cdc20} activity *in vitro* (Fang *et al*, 1998; Tang *et al*, 2001; Fang, 2002). Furthermore, Mad2 and BubR1 (together with Bub3) can coexist in the same complex, called the mitotic checkpoint complex (MCC) (Sudakin *et al*, 2001), but distinct Cdc20 complexes comprising either Mad2 or BubR1:Bub3 have also been described (Tang *et al*, 2001; Fang, 2002). Recent evidence suggests that, during checkpoint action, KT-activated Mad2 entraps Cdc20 first, thereby priming it for subsequent binding of BubR1:Bub3. The latter proteins would then sequester Cdc20 through a complex in which Mad2 is substoichiometric (Nilsson *et al*, 2008; Kulukian *et al*, 2009).

While the exact nature of the anaphase inhibitor remains a matter of debate, our understanding of the role of Mad2 in SAC signalling has benefited greatly from structural analysis (Luo *et al*, 2002, 2004; Sironi *et al*, 2002; Mapelli *et al*, 2007). The Mad2 protein can in fact adopt two distinct natively folded states: ‘open’ (or ‘N1’) and ‘closed’ (or ‘N2’) (O- and C-Mad2, respectively). The two conformers differ in the orientation of a C-terminal β -sheet that in the closed conformation surrounds the polypeptide chain of a Mad2 interaction partner (or ‘ligand’) in a structure reminiscent of a safety belt (Sironi *et al*, 2002). Best-known ligands of C-Mad2 are Mad1 and Cdc20. Central to a prevailing model of SAC signalling, the so-called ‘template model’, is the ability of Mad2 to dimerize asymmetrically, that is O-Mad2 can bind C-Mad2 (De Antoni *et al*, 2005; Mapelli *et al*, 2006, 2007). Moreover, reconstitution experiments have shown that a complex of Mad1:C-Mad2 can, through Mad2 asymmetric dimerization, enhance the ability of O-Mad2 to bind Cdc20, thereby generating a structurally equivalent C-Mad2:Cdc20 complex (De Antoni *et al*, 2005; Nasmyth, 2005; Vink *et al*, 2006; Kulukian *et al*, 2009; Lad *et al*, 2009). Some aspects of this template model find experimental support also in the cellular environment: Mad1 clearly is the KT receptor of Mad2 (Chen *et al*, 1998; Luo *et al*, 2002; Martin-Bluesma *et al*, 2002) and interfering with Mad1 affects the ability of Mad2 to bind Cdc20 (Hwang *et al*, 1998; Hardwick *et al*,

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Received: 24 March 2011; accepted: 27 June 2011; published online: 19 July 2011

2000; Fraschini *et al*, 2001). Furthermore, FRAP experiments performed in mammalian cells revealed a biphasic recovery after photobleaching of KT-associated Mad2, indicating the existence of two distinct Mad2 populations (Shah *et al*, 2004). While one population showed a slow turnover reminiscent of the recovery kinetics of Mad1 (Shah *et al*, 2004), the other Mad2 population turned over much faster, similar to the behaviour of Cdc20 at KTs (Howell *et al*, 2004).

Chromosome bi-orientation leads to SAC silencing and this in turn allows anaphase onset. While several pathways involved in SAC silencing have been described, two are thought to be crucial in mammalian cells. The first is based on the dynein-dependent 'stripping' from KTs of SAC components and other proteins, notably Spindly, upon microtubule (MT) attachment (Howell *et al*, 2001; Gassmann *et al*, 2010). The second is based on a binding partner of Mad2, known as p31comet, which associates selectively with the dimerization interface of C-Mad2 *in vitro* (Xia *et al*, 2004; Mapelli *et al*, 2007; Yang *et al*, 2007). Depletion of p31comet from cells interferes with efficient recovery from a SAC-dependent arrest, whereas overexpression of p31comet causes a SAC override (Habu *et al*, 2002; Xia *et al*, 2004; Yang *et al*, 2007). Although these data strongly implicate p31comet in SAC silencing, a mechanistic understanding of its action remains elusive. In particular, it is unknown how the p31comet:Mad2 interaction is regulated in time and space *in vivo*.

At present, the template model is supported by several lines of evidence, but direct experimental proof for its operation within cells is lacking. Furthermore, some observations, particularly the purported Mad1-independent function of Mad2 in regulating mitotic timing (Meraldi *et al*, 2004), do not readily find a mechanistic explanation in the template model. Here, we characterize a monoclonal antibody (mAb) raised against human Mad2 and demonstrate that it specifically recognizes the dimerization interface of the closed conformer of Mad2. By using this mAb we show, first, that Mad2 requires the association with Mad1 to adopt the closed conformation and, second, that the Mad1:C-Mad2 complex undergoes spatio-temporal regulation by p31comet-dependent 'capping' of C-Mad2's dimerization interface. Finally, we demonstrate that neutralization of the Mad1:C-Mad2 complex by microinjection of antibodies directed against either C-Mad2 or Mad1 abolishes the SAC and causes mitotic acceleration, thus challenging the notion that cytoplasmic Mad2 controls mitotic timing independently of Mad1. Collectively, our study provides direct *in vivo* evidence to support the model that the Mad1:C-Mad2 complex acts as a template at KTs to trigger SAC signalling in human cells.

Results

A mAb specific for C-Mad2

The mAbs represent powerful tools to visualize and characterize physiologically and/or pathophysiologically relevant conformers of particular proteins (see for example, Gannon *et al*, 1990; Korth *et al*, 1997). The mAb 107–276 readily recognizes human Mad2 by immunoprecipitation (IP) and immunofluorescence, but it does not detect the denatured protein in western blots (WBs) (Chan *et al*, 2009). In addition, an attempt to identify the epitope of this mAb using a 12-mer peptide array encompassing the entire Mad2 sequence failed to reveal reactivity, suggesting that this mAb

recognizes a conformational rather than a linear epitope (Supplementary Figure S1A–C). To determine whether the mAb 107–276 displays a preference for a specific Mad2 conformer, we purified His-tagged Mad2 from *Escherichia coli* in either its wild-type form (Mad2^{WT}) or as mutants (Mad2^{V193N} and Mad2^{L13Q}, respectively) that are known to mimic the two distinct conformers, O-Mad2 and C-Mad2 (Mapelli *et al*, 2007; see also Supplementary Figure S1G and H for a summary of the mutants' characteristics). In IP, the mAb displayed increased reactivity towards C-Mad2 when compared with Mad2^{WT} or O-Mad2 (Figure 1A). These results demonstrate that the mAb 107–276 is specific for the closed conformer of Mad2; therefore, it will hereafter be referred to as 'CM2²⁷⁶' (C-Mad2, clone 276). Note that a Mad2 polyclonal antibody that did not display preference for any Mad2 conformer in denaturing conditions (Figure 1A, lower panel) was used for all WB experiments throughout this work.

CM2²⁷⁶ recognizes C-Mad2 on its dimerization interface

A prediction from the above results is that CM2²⁷⁶ should be able to coprecipitate the known C-Mad2 interaction partners. Therefore, we asked whether the CM2²⁷⁶ can coprecipitate Mad1, Cdc20 and/or p31comet from a human cell lysate. Both Mad1 and Cdc20 were enriched in the immune complexes obtained after immunoprecipitating C-Mad2 from SAC-arrested HeLaS3 cells, whereas p31comet was not (Figure 1B). We also used CM2²⁷⁶ to immunoprecipitate C-Mad2 from a lysate of cells synchronized in metaphase by the proteasome inhibitor MG132. Again, p31comet was not detected in CM2²⁷⁶ immune complexes, although it was present in immunoprecipitates that were prepared using a polyclonal Mad2 antibody (Figure 1C; see also Supplementary Figure S1D for a more extensive panel of WBs). These results demonstrate that CM2²⁷⁶ is unable to coimmunoprecipitate p31comet with Mad2, even though a Mad2:p31comet complex is present in metaphase-arrested cells.

p31comet is known to interact with C-Mad2 through direct contact with the dimerization interface of Mad2 (Mapelli *et al*, 2006; Yang *et al*, 2007). Thus, we asked whether CM2²⁷⁶ competes with p31comet for C-Mad2 binding. Specifically, we performed GST pull-down assays, using GST-Cdc20^{111–138} that had been preadsorbed on glutathione-sepharose beads (GSH) and bound to His-tagged WT-Mad2 (Mad2^{WT}), thereby generating a C-Mad2:GST-Cdc20^{111–138} complex (Mapelli *et al*, 2006). As expected, this complex readily pulled down both CM2²⁷⁶ and His-tagged p31comet, provided that the two proteins were added separately (Figure 1D, lanes 1 and 2). In striking contrast, coinubation of CM2²⁷⁶ with increasing concentrations of p31comet decreased the amount of CM2²⁷⁶ that was bound by C-Mad2 (Figure 1D, lanes 3–5), clearly demonstrating competition between p31comet and CM2²⁷⁶ for binding to C-Mad2. Attesting to the specificity of this competition, increasing concentrations of the unrelated protein His-Spindly^{1–444} (Chan *et al*, 2009) did not affect the association of CM2²⁷⁶ to C-Mad2 in this assay (Supplementary Figure S1E). One prediction of the observed competition between p31comet and CM2²⁷⁶ is that the antibody should interfere with Mad2 conformational dimerization (Mapelli *et al*, 2006, 2007; Yang *et al*, 2007). Indeed, coinubation of CM2²⁷⁶ with Mad2^{AC}, known to adopt the open conformation

Figure 1 Biochemical characterization of the C-Mad2 mAb (CM2²⁷⁶). (A) Purified Mad2^{WT} (WT), Mad2^{L13Q} (closed) and O-Mad2^{V193N} (open) were immunoprecipitated (IP) with CM2²⁷⁶. Inputs and IPs were analysed by Coomassie Brilliant Blue staining (CBB) and WB using a rabbit polyclonal Mad2 antibody. IgG HC and LC indicate heavy and light IgG chains, respectively. (B) IPs were performed with unspecific IgGs or CM2²⁷⁶ from HeLaS3 lysate obtained from cells arrested with nocodazole. (C) IPs were performed from HeLaS3 cells released from nocodazole for 2.5 h in the presence of MG132 using rabbit unspecific IgGs and a rabbit Mad2 antibody (IP (rabbit)) or with mouse unspecific IgGs and CM2²⁷⁶ (IP (mouse)). Input and IPs in (B, C) were analysed by WB using the indicated antibodies. (D) Purified CM2²⁷⁶, p31comet or a mixture of constant amount of Mad2 mAb with increasing concentrations of p31comet were incubated with Mad2:GST-Cdc20¹¹¹⁻¹³⁸ complex preadsorbed on GSH beads. (E) Purified CM2²⁷⁶, Mad2^{ΔC} or a mixture of Mad2^{ΔC} with increasing concentrations of CM2²⁷⁶ were treated as described in (D). Asterisks in (D, E) indicate a contaminant of the GST-Cdc20¹¹¹⁻¹³⁸ preparation with electrophoretic mobility similar to the IgG light chain. Inputs and GST pull downs in (D, E) were analysed by Coomassie Brilliant Blue staining. The arrow and the circle in (D, E) mark the species used at constant or increasing concentrations, respectively. (F) Purified Mad2^{ΔC} was added to Mad2:GST-Cdc20¹¹¹⁻¹³⁸ complex adsorbed on GSH beads, following preincubation with buffer (–) or CM2²⁷⁶ (+). GST pull downs were analysed by Coomassie Brilliant Blue staining. The arrow marks Mad2^{ΔC}. (G) Purified Mad2^{WT} (WT) and Mad2^{RQ} (R133E–Q134A) were immunoprecipitated with CM2²⁷⁶. Inputs and IPs were analysed by Coomassie Brilliant Blue staining. (H) Schematic representation of the CM2²⁷⁶ epitope.

(Supplementary Figure S1G), also resulted in a competitive behaviour in that CM2²⁷⁶ abolished the ability of Mad2^{ΔC} to bind to C-Mad2:GST-Cdc20¹¹¹⁻¹³⁸ (Figure 1E, lanes 3–5). In contrast, equal concentrations of a Mad1 mAb did not interfere with the binding between Mad2^{ΔC} and C-Mad2, demonstrating specificity of this competition (Supplementary Figure S1F). Binding of Mad2^{ΔC} was also prevented upon prior adsorption of CM2²⁷⁶ to C-Mad2:GST-Cdc20¹¹¹⁻¹³⁸ beads (Figure 1F). To further demonstrate that CM2²⁷⁶ binds to the C-Mad2 dimerization interface, we also tested its ability to recognize a Mad2 protein that carries the double mutation R133E–Q134A (Mad2^{RQ}), which is known to affect residues crucial for Mad2 dimerization and p31comet binding (De

Antoni *et al*, 2005; Yang *et al*, 2007) (Supplementary Figure S1G). As shown in Figure 1G, Mad2^{RQ} was barely able to bind to CM2²⁷⁶ when compared with Mad2^{WT}. The most likely interpretation of these data is that CM2²⁷⁶ recognizes a conformational epitope on the dimerization interface of C-Mad2 and that this epitope is critical for both conformational dimerization and p31comet binding (Figure 1H).

p31comet masks the presence of C-Mad2 at nuclear pore complexes and spindle poles

Mad1 is known to form a constitutive protein complex with Mad2 throughout the cell cycle (Chen *et al*, 1998; Campbell *et al*, 2001; De Antoni *et al*, 2005). The two proteins also

display identical localizations. They decorate the nucleoplasmic side of the nuclear pore complex (NPC) during interphase (Campbell *et al*, 2001) and then relocate to KT's upon mitotic entry. Upon KT-MT attachment, they are 'stripped' from KT's in a dynein and MT-dependent manner and transiently accumulate at spindle poles (Li and Benezra, 1996; Gorbisky *et al*, 1998; Howell *et al*, 2001). These exact localization patterns were seen when endogenous Mad1 was stained with a mAb in HeLaS3 cells (Figure 2A; see Supplementary Figure S2 for characterization of Mad1 mAbs). However, contrary to expectation, costaining of the same cells with

CM2²⁷⁶ revealed only partial colocalization with Mad1. Although CM2²⁷⁶ staining revealed clear KT localization from prophase to prometaphase, C-Mad2 could barely be detected by this antibody at NPCs and spindle poles (Figure 2A; see Supplementary Figure S3 for results using methanol as alternative cell fixation method). One possible explanation for this result is that Mad2 adopts the open conformation at NPCs and spindle poles. Alternatively, one or more proteins might mask the dimerization interface of C-Mad2 at NPCs and spindle poles. As p31comet localizes to both NPCs (Tighe *et al*, 2008; A Musacchio, personal

Figure 2 Subcellular localization of C-Mad2. **(A)** HeLaS3 cells arrested with thymidine for 24 h were released for 10 h and fixed with PTEMF. After fixation, C-Mad2 was stained with CM2²⁷⁶, followed by secondary antibody incubation (red). After extensive washing, Mad1 was costained with a Mad1 mAb directly coupled to Alexa Fluor 488 (green), DNA was visualized with DAPI (blue). White arrows indicate spindle pole staining. **(B)** Cells were transfected with the indicated siRNA oligonucleotides for a total of 48 h, and synchronized as described in **(A)**. Lysates were obtained and analysed by WB with the indicated antibodies. **(C, D)** Cells transfected with the indicated siRNA oligonucleotides as in **(B)** were treated as described in **(A)**. Scale bars = 10 μ m. **(E–G)** Box-and-whisker plots showing the average pixel intensities at single KT's expressed as the Mad1/CREST fluorescence ratio in **(E)**, the CM2²⁷⁶/Mad1 ratio in **(F)** and the LAP^{CFP}-mouse-Mad2 (LAP-mMad2)/Mad1 ratio in **(G)** either in Gl2 or in p31comet-1 siRNA-treated cells. a.u. = arbitrary units, $n \geq 80$ KT's from four independent cells for **(E, F)** and $n \geq 180$ KT's from eight independent cells for **(G)**. Lower and upper whiskers represent 10th and 90th percentiles, respectively.

communication) and the mitotic spindle (Habu *et al*, 2002), and we have previously demonstrated that p31comet can mask the CM2²⁷⁶ epitope (Figure 1D), we asked whether depletion of p31comet could restore the ability of CM2²⁷⁶ to detect Mad2 at NPCs and spindle poles. Indeed, depletion of p31comet by two independent siRNA oligonucleotides readily allowed the detection of C-Mad2 by CM2²⁷⁶ at both NPCs and spindle poles (Figure 2C and D, respectively; for efficiency of p31comet depletion see Figure 2B). To substantiate this conclusion, we also used a HeLa cell line expressing LAP^{GFP}-mouse-Mad2 (Poser *et al*, 2008; Hubner *et al*, 2010), which allowed us to study the localization of Mad2 through the monitoring of GFP fluorescence. As predicted, GFP fluorescence at the nuclear envelope and at spindle pole staining was indistinguishable, regardless of the presence or absence of p31comet (Supplementary Figure S4A–C).

Most interestingly, we also observed that p31comet depletion caused a detectable increase in CM2²⁷⁶ signal at KTJs, without affecting the level of resident Mad1:C-Mad2 complex (Figure 2D and E). Quantification (Figure 2F) indicates that p31comet masks ~50% of C-Mad2 at KTJs. Furthermore, a significant increase in GFP signal at KTJs could be observed in the cell line expressing LAP^{GFP}-mouse-Mad2 (Figure 2G), suggesting that additional Mad2, almost certainly in the open conformation, had been recruited upon depletion of p31comet. Taken together, these data indicate that Mad2 can adopt the closed conformation at multiple subcellular sites,

but that the dimerization interface of Mad2 undergoes masking by p31comet at NPCs, spindle poles and, partially, also at KTJs. The data also indicate that unmasking of C-Mad2 leads to recruitment of O-Mad2 selectively at KTJs, implying that asymmetric dimer formation is differentially regulated at KTJs, NPCs and spindle poles.

p31comet localizes to KTJs in a C-Mad2-dependent manner

The observation that a sizeable population of KTJ-associated C-Mad2 could be unmasked by depletion of p31comet predicts that p31comet associates with KTJs already during prometaphase, when the SAC is active, and that this KTJ association should depend on C-Mad2. To explore this point, we expressed Myc-p31comet in its wild-type form or as a version carrying two point mutations (Q83A–F191A, abbreviated as p31comet^{QAFA}) that are known to abolish binding to C-Mad2 (Yang *et al*, 2007). Importantly, the use of a SV40 promoter allowed us to express p31comet at subendogenous levels (Figure 3A). Following cytosolic pre-extraction, Myc-p31comet^{WT}, but not Myc-p31comet^{QAFA}, was readily visualized at C-Mad2-positive KTJs (Figure 3B). In addition, depletion of endogenous Mad2 by siRNA completely prevented Myc-p31comet^{WT} to localize to KTJs (Figure 3C). Taken together, these data show that p31comet can localize at KTJs during an active SAC and that the Mad1:C-Mad2 complex acts as its main KTJ receptor.

Figure 3 p31comet localizes at KTJs in a Mad2-dependent manner. (A) HeLaS3 cells were transfected with the indicated SV40-Myc expression vectors for a total of 36 h. At 12 h after transfection, cells were arrested with thymidine for 14 h and subsequently released for 10 h. Lysates were obtained and analysed by WB with the indicated antibodies. Bands corresponding to endogenous and ectopically expressed p31comet are indicated ('endo-p31comet' and 'Myc-p31comet', respectively). (B) Cells transfected with the indicated expression vectors were treated as described in (A). CM2²⁷⁶ and rabbit-Myc antibodies were detected by secondary antibody staining (red and green, respectively), whereas the Mad1 mAb was directly coupled to Alexa Fluor 647 (displayed in blue). (C) Cells transfected with either G12 or Mad2 siRNA for a total of 48 h were subsequently transfected with the SV40-Myc-p31comet^{WT} construct, synchronized and treated as described in (B). Scale bars = 10 μ m.

Only liganded Mad2 adopts the closed conformation throughout the cell cycle

The template model predicts not only that Mad2 adopts two distinct conformations in the cell, open and closed, but also that O-Mad2 should freely diffuse in the cytoplasm and, by definition, be devoid of ligand, whereas C-Mad2 should tightly bind Mad1 and therefore be a resident component of unattached KTs. Furthermore, during SAC signalling, the Mad1:C-Mad2 complex is predicted to exert a catalytic function by triggering the conversion (through asymmetric dimerization) of O-Mad2 to C-Mad2 (with Cdc20 as ligand). Although this model is appealing and strongly supported by structural data (Mapelli and Musacchio, 2007; Luo and Yu, 2008), the lack of tools to discriminate between O-Mad2 and C-Mad2 has so far precluded the analysis of Mad2 conformation in a cellular context. To monitor the two-state behaviour of Mad2 in cells, we fractionated cell lysates by size exclusion chromatography (SEC) and analysed each fraction for the presence of C-Mad2, using CM2²⁷⁶ for IP (Figure 4A). In order to analyse transitions between interphase and mitosis as well as cells with both an active and an inactive SAC, we synchronized HeLaS3 cells in G1/S phase, prometaphase and metaphase (by addition of thymidine, nocodazole and MG132, respectively; Figure 4B). The use of a Superdex 200 column resulted in a Mad2 elution profile that showed two peaks of comparable intensity; these reflect Mad2 associated with its interaction partners (>440 kDa) and monomeric Mad2 (ca. 25 kDa), respectively (Figure 4C, 'TCAP' panels; see also Materials and methods). CM2²⁷⁶ IP on all fractions invariably revealed the ability of this mAb to recognize Mad2 bound to its interaction partners, but not free Mad2 (Figure 4C, 'CM2²⁷⁶ IP' panels). These data confirm that Mad2 adopts the closed conformation when in complex with other proteins. Moreover, they indicate that free Mad2 adopts the open conformation at all cell cycle stages examined.

Theoretically, one could argue that the free Mad2 could actually be in the closed conformation but not be recognized by CM2²⁷⁶, due to epitope masking by p31comet. However, this is clearly not the case as p31comet did not extensively comigrate with free Mad2 (Figure 4D). We also note that the elution profile of p31comet did not show major changes during the different cell cycle stages analysed (Figure 4D). At first glance, this may appear surprising in view of our cell biological data (shown above), which indicate that p31comet undergoes cell-cycle-regulated interactions with Mad1:C-Mad2 complexes. Although the absence of p31comet in the CM2²⁷⁶ IPs is readily explained by antibody competition for the p31comet-binding site (see Figure 1D and H), it is more difficult to explain the absence of p31comet in the high molecular weight fractions of total cell lysates. We interpret this to indicate that only a very minor fraction of the total cellular p31comet interacts with Mad1:C-Mad2 complexes at any given stage. In support of this conclusion, semi-quantitation of cellular protein levels indicates that p31comet is approximately twice as abundant as total Mad2 (Supplementary Figure S5B).

WBs for the other known Mad2 ligands revealed that Mad1 comigrated with C-Mad2 and could be coimmunoprecipitated by CM2²⁷⁶ throughout the cell cycle (Figure 4E). In stark contrast, Cdc20 migrated as a monomeric species in interphase and did not display any interaction with C-Mad2

(Figure 4F, upper panels), arguing against the existence of a sizeable pool of MCC during interphase. During SAC activation, Cdc20 shifted to the higher molecular weight fractions, where it clearly interacted with C-Mad2 (Figure 4F, middle panels). Upon SAC silencing, however, the bulk of Cdc20 was again released as a monomeric entity and only a minor population, still engaged in protein complexes, could be coimmunoprecipitated by CM2²⁷⁶ (Figure 4F, lower panels). Interestingly, the association of C-Mad2 with Cdc20 during prometaphase was not reflected in a major change in the overall elution profile of total Mad2 (Figure 4C), consistent with the emerging notion that the inhibitory action of Mad2 on Cdc20 is likely to be catalytic rather than stoichiometric (Nilsson *et al*, 2008; Kulukian *et al*, 2009). A similar experiment performed with a Superose 6 column also revealed the presence of C-Mad2 in both the MCC and the APC/C + MCC complexes (Supplementary Figure S5C–E). Collectively, our results show that liganded Mad2 adopts the closed conformation in human cells, whereas free Mad2 remains in the open conformation during the cell cycle (Figure 4G; see also Luo *et al*, 2004).

Mad1 is required for the formation of C-Mad2 in interphase

Our data show that, within cells, Mad1 is a constitutive ligand of C-Mad2, whereas Cdc20 is entrapped into Mad2's safety belt only during checkpoint activation (Figure 4). As C-Mad2 is the energetically favoured conformer *in vitro* (Luo *et al*, 2004), we asked whether Mad2 can possibly adopt the closed conformation *in vivo*, in the absence of any known ligand. To this end, we used siRNA to deplete Mad1 from interphase-arrested cells, in which Mad1 is the only known ligand of C-Mad2, before samples were analysed using the assay described above. When compared with the Gl2 control, Mad1 siRNA caused a significant (albeit not complete) reduction in Mad1 protein levels (Figure 5A, B and E). Under these conditions, a marked shift of Mad2 from the liganded population to the free population was observed (Figure 5A, B and E), confirming that Mad1 is the major binding partner of Mad2 in interphase cells. Importantly, while CM2²⁷⁶ was able to immunoprecipitate the Mad1:C-Mad2: complex, but not free Mad2, from Gl2 control cells (Figure 5C–E), the amount of C-Mad2 that could be precipitated by CM2²⁷⁶ from Mad1-depleted cells was decreased in proportion to the reduction in Mad1 levels, without a corresponding increase of free Mad2 in the CM2²⁷⁶ IP (Figure 5D and E). These data clearly demonstrate that the association with Mad1 is required for interphase Mad2 to adopt the closed conformation; furthermore, they suggest that, *in vivo*, C-Mad2 does not accumulate to significant levels in the absence of ligand (Figure 5F and G).

Conformational dimerization of Mad2 is essential to initiate and maintain a SAC-dependent arrest

The key prediction made by the template model is that C-Mad2 is required for the catalytic activation of a cytosolic pool of O-Mad2 and that this conversion is in turn indispensable for SAC signalling. The ability of CM2²⁷⁶ to interfere with Mad2 conformational dimerization afforded a unique opportunity to test this model within the cellular context. In a series of experiments, mAbs were microinjected into HeLaS3 stably expressing histone H2B-GFP and progression through

Figure 4 Distribution of C-Mad2 among 'free' and 'bound' pools of the protein. **(A)** Schematic representation of the approach followed to study the distribution of C-Mad2. Synchronized HeLaS3 cells were lysed and fractionated by SEC. SEC was performed with a Superdex 200 16/60 column, followed by collection of 4 ml fractions through the 120-ml of elution volume. Fourteen fractions covering size ranges from the upper limit of the column to 13.7 kDa were further processed in parallel by TCA precipitation (TCAP) and CM2²⁷⁶ IP. Resulting samples were analysed by WB. **(B)** Timeline of the synchronization used for experiments shown in **(C–E)**; 'MG132' cells were first arrested in thymidine for 24 h and were incubated in the presence of nocodazole for 12 h after release, collected by mitotic shake off and released for 2.5 h in the presence of MG132. 'thymidine' and 'nocodazole' cells were harvested following thymidine and nocodazole block, respectively. **(C–F)** WBs on samples obtained from TCA precipitations (TCAP) and CM2²⁷⁶ IP on fractions obtained as described in **(A)**, following synchronization as described in **(B)**. WBs were performed with rabbit Mad2 polyclonal antibody **(C)**, p31comet antibody **(D)**, Mad1 mAb **(E)** and Cdc20 antibody **(F)**. **(G)** Schematic summary of the results displayed in **(C–F)**.

mitosis was monitored by time-lapse video microscopy. We first asked whether C-Mad2 is required to initiate SAC signaling. To this end, cells were injected in interphase and treated with nocodazole before progress through mitosis was monitored (Figure 6A). As expected, the microinjection of a Myc mAb, used as negative control, did not interfere with the

ability of HeLaS3 cells to respond to nocodazole, as all injected cells underwent mitotic arrest for at least 6 h without signs of apoptosis (Figure 6B and C; Supplementary Movie S1). In stark contrast, the majority of the CM2²⁷⁶-injected cells escaped the nocodazole block in < 1 h, resulting in cells with decondensed chromatin and aberrantly shaped nuclei,

Figure 5 Requirement of Mad1 for C-Mad2 formation in interphase. (A) SEC analysis of a lysate obtained from HeLaS3 cells treated with Gl2 siRNA for 60 h and arrested with thymidine for 24 h. Samples were concentrated by TCA precipitation (TCAP). WBs were performed with the indicated antibodies. (B) Samples obtained as in (A) but following Mad1 siRNA-mediated depletion. (C, D) Samples obtained by CM2²⁷⁶ IP against the same fractions used in (A, B), respectively. WBs were performed with indicated antibodies. (E) Fractions highlighted as large and small in (A–D) were pooled and WBs were performed with the indicated antibodies. (F) Schematic representation of the Mad2 subcomplexes found in interphase: Mad2 is either bound to Mad1, adopting the closed conformation or free, adopting the open conformation. (G) In the absence of Mad1, Mad2 cannot adopt the closed conformation during interphase, and the bulk is thereby released as open free Mad2.

a phenotype typical of SAC override (Figure 6B and C; Supplementary Movie S2). Importantly, the microinjection phenotype caused by CM2²⁷⁶ injection depended exclusively on C-Mad2 recognition, as coinjection of the antibody with Mad2^{L13Q} but not Mad2^{V193N} (mimicking C- and O-Mad2, respectively), abolished the observed phenotype (Supplementary Figure S6). Microinjection of a Mad1 mAb (117–468; Supplementary Figure S2), performed as a positive control, resulted in a nearly identical phenotype (Figure 6B and C; Supplementary Movie S3). These results strongly indicate that the conformational dimerization of Mad2 is required for SAC signalling, as predicted by the template model and supported by experiments conducted in budding yeast (Nezi *et al*, 2006).

Next, we asked whether Mad2 conformational dimerization is also required to maintain an already established SAC arrest. This question was of particular interest, as several lines of recent evidence indicate that Mad2 acts epistatically upstream of BubR1/Mad3 (Hardwick *et al*, 2000; Burton and Solomon, 2007; King *et al*, 2007; Nilsson *et al*, 2008; Kulukian *et al*, 2009). To examine this issue, we injected CM2²⁷⁶ into mitotic HeLaS3 cells in which the SAC had been activated by treatment with nocodazole and analysed the injected cells 20, 40 and 60 min later by fluorescence microscopy (Figure 7A). Already 20 min after CM2²⁷⁶ injection, levels of Cyclin B1 were decreased to background levels in approximately half of the cells, and by 60 min all injected cells had overcome the nocodazole-induced arrest (Figure 7B and C), while injection of Myc mAb for control did not trigger a SAC override, as

expected (Figure 7B and C). We have also analysed the kinetics of SAC override in response to microinjection of the anti-Mad1 mAb. This antibody triggered an even faster SAC override than CM2²⁷⁶ (Figure 7B and C). These results demonstrate, first, that C-Mad2 (and hence conformational dimerization) is required to maintain an already established SAC-dependent arrest, supporting and extending previous data obtained with a polyclonal Mad2 antibody (Gorbsky *et al*, 1998). They do not contradict an important role for BubR1 in APC/C^{Cdc20} inhibition, but demonstrate a continuous requirement for C-Mad2 for sustaining this inhibition. Second, they also bear on a model of SAC signalling that invokes a contribution of an autocatalytic loop based on cytosolic C-Mad2 (De Antoni *et al*, 2005). Specifically, our side-by-side comparison of the consequences of anti-Mad1 mAb and CM2²⁷⁶ injection argues that cytosolic C-Mad2 alone cannot provide sufficient C-Mad2:Cdc20 to maintain a SAC arrest.

The Mad1:C-Mad2 complex is required to regulate proper mitotic timing

In addition to being an essential component of the SAC, Mad2 has been proposed to constitute a key component of a mitotic timer operating in the cytosol (Meraldi *et al*, 2004). In support of this notion, the siRNA-mediated knockdown of Mad2 and BubR1 caused not only SAC silencing but also a striking acceleration of mitotic progression, whereas the knockdown of other Mad or Bub components was reported to abolish the

Figure 6 C-Mad2 is required for initiating a SAC-dependent arrest. **(A)** Schematic representation of the microinjection protocol followed to assess the ability of cells to respond to nocodazole upon CM2²⁷⁶ injection. Interphase HeLaS3 stably expressing histone H2B-GFP cells were subjected to microinjection 7 h after thymidine release and analysed by time-lapse video microscopy in the presence of nocodazole. **(B)** Movie stills from representative cells injected with the indicated mAbs. Time in h:min is indicated. *T* = 0 was defined as the time point at which NEBD became evident. Scale bars = 10 μm. **(C)** Histogram showing cells microinjected with the indicated mAbs, subdivided into the displayed categories according to the time elapsed between NEBD and nocodazole override (SAC override). Percentage of cells is shown (*n* = total number of cells counted).

SAC without accelerating the traverse of mitosis (Meraldi *et al*, 2004).

A mechanistic understanding of the proposed KT-independent mitotic timer is presently elusive, but the fact that Mad1 was reportedly dispensable (Meraldi *et al*, 2004) suggested the non-catalytic generation of a C-Mad2:Cdc20 complex. To clarify the relationship between the proposed Mad2-dependent mitotic timer and the SAC in human cells, we took advantage of CM2²⁷⁶ and asked whether interfering with conformational dimerization (and therefore C-Mad2 catalysis) would cause a similar mitotic acceleration, as the generalized Mad2 knockdown. We injected interphase HeLaS3 stably expressing histone H2B-GFP with either CM2²⁷⁶ or

Myc mAb, as control, and analysed their progression through an unperturbed mitosis by time-lapse video microscopy (Figure 8A). Injection of Myc mAb (or buffer alone) caused a noticeable delay in the elapsed time between nuclear envelope breakdown (NEBD) and anaphase onset (54 ± 8.1 and 69 ± 6.1 min in average $\pm$ s.d., respectively), when compared with control non-injected cells (41.7 ± 3.7 min) (Figure 8B and C; Supplementary Movies S4–S6), most likely reflecting the cellular stress caused by the microinjection procedure. More importantly, injection of CM2²⁷⁶ led to a drastic mitotic acceleration (22.4 ± 1.5 min) (Figure 8B and C; Supplementary Movie S7); in addition, CM2²⁷⁶-injected cells invariably showed signs of chromosome missegregation

Figure 7 The Mad1:C-Mad2 complex is required for maintaining a SAC-dependent arrest. **(A)** Schematic representation of the protocol followed to assess the capability of cells to maintain an already established SAC-dependent arrest upon microinjection of CM2²⁷⁶ and Mad1 mAb. Asynchronous HeLaS3 cells were treated with nocodazole for 5 h and mitotic cells were selectively microinjected with the mAbs. After injection, cells were incubated for 20, 40 or 60 min in the presence of nocodazole and fixed with PTEMF. Cyclin B1 was stained with a mAb directly coupled to Alexa Fluor 488. **(B)** Representative cells treated as described in **(A)**. Cyclin B1 (green), DNA was visualized with DAPI (blue) and the injection marker Texas Red dextran (red) are displayed. Scale bar = 10 μ m. **(C)** Histogram showing cells microinjected with the indicated mAbs, subdivided into the displayed categories according to the absence (–) or presence (+) of Cyclin B1 staining. Percentage of cells is indicated (n = total number of cells counted).

indicative of SAC abrogation (i.e. chromosome bridges and/or lagging chromosomes) (Figure 8B). These results clearly show that conformational dimerization of Mad2 is also involved in regulating mitotic timing.

From the perspective of the proposed timer model, allegedly dependent on Mad2 but not Mad1, the above results create a conundrum. It implies that either C-Mad2 can be generated in a Mad1-independent manner or, alternatively, Mad1 is also part of the mitotic timer mechanism. To distinguish between these possibilities, we used the Mad1 mAb, which was able to neutralize the SAC (Figure 6; Supplementary Movie S3), to carry out microinjection experiments. Remarkably, microinjection of this anti-Mad1 mAb led to a striking acceleration of unperturbed mitosis (19.1 ± 1.2 min), causing also a strong chromosome missegregation phenotype (Figure 8B and C; Supplementary Movie S8), similar to the results obtained when injecting CM2²⁷⁶.

This indicates that Mad1 also contributes to mitotic timing regulation.

In order to address to which extent the regulation of mitotic timing relies on the formation of an APC/C inhibitor during interphase, we undertook two different but complementary approaches. First, antibodies were microinjected into cells that had been arrested at the G2/M boundary with the Cdk1 inhibitor RO-3306 (Vassilev *et al*, 2006), and mitotic progression was then monitored following inhibitor wash out (Supplementary Figure S7A). The injection of either CM2²⁷⁶ or the anti-Mad1 mAb caused a striking mitotic acceleration (NEBD-to-anaphase onset; 24.4 and 22.3 min on average, respectively) when compared with non-injected cells (68.5 min on average) (Figure 8D; Supplementary Figure S7B). Second, we took advantage of the fact that the SAC kinase Mps1, amenable to chemical inhibition and required for Mad1:C-Mad2 loading to KTs, has also been recently

Figure 8 The Mad1:C-Mad2 complex is required for regulating mitotic timing. **(A)** Schematic representation of the microinjection protocol followed to analyse the progression through an unperturbed mitosis after mAb microinjection. Interphase HeLaS3 stably expressing histone H2B-GFP cells were subjected to microinjection 7 h after thymidine release and analysed by time-lapse video microscopy. **(B)** Movie stills from representative cells injected with the indicated mAbs as described in **(A)**. Control cells correspond to non-injected cells. Time in h:min is indicated. $T=0$ was defined as the time point at which NEBD became evident. Scale bar = 10 μ m. **(C)** Box-and-whisker plot showing the elapsed time (min) between NEBD and anaphase onset for individual cells microinjected as described in **(A)**. Analysis performed on >40 cells per condition, from three independent experiments. **(D)** Box-and-whisker plot showing the elapsed time (min) between NEBD and anaphase onset for individual cells microinjected as described in Supplementary Figure S7. Analysis performed on ≥ 10 cells. Lower and upper whiskers in **(C, D)** represent 10th and 90th percentiles, respectively.

implicated in the regulation of mitotic timing (Hewitt *et al*, 2010; Maciejowski *et al*, 2010; Santaguida *et al*, 2010; Sliedrecht *et al*, 2010). Specifically, we monitored the timing of mitotic progression in cells that experienced an Mps1

inhibition already during S, G2 and M phase or only during M phase (Supplementary Figure S8A). Remarkably, Mps1 inhibition led to an indistinguishable mitotic acceleration, regardless of the timing of inhibitor addition (Supplementary

Figure S8B and C). These results lend no support to the idea that Mps1 affects the duration of mitosis independently of the SAC.

Taken together, the above data identify the KT Mad1:C-Mad2 complex as a prominent regulator of mitotic timing in human cells and they call for a reconsideration of the distinction between SAC and mitotic timer in human cells.

Discussion

In this study, we have used a conformation-specific mAb (CM2²⁷⁶) to study mechanistic aspects of SAC signalling in living human cells. We demonstrate that CM2²⁷⁶ selectively recognizes one of the two Mad2 conformers, namely C-Mad2. This powerful tool provided a unique opportunity to probe Mad2 conformation and directly validate several aspects of the template model for SAC signalling in a cellular context. In particular, the CM2²⁷⁶ antibody allowed us to reveal a role for p31comet in capping C-Mad2 at particular subcellular locations, and to demonstrate that Mad1 is required for the generation of C-Mad2 *in vivo*, thus providing strong support for the template model. Finally, we demonstrate that mitotic timing also depends on the Mad1:C-Mad2 template. This latter observation suggests that, from a mechanistic perspective, the SAC and the proposed mitotic timer are closely related if not identical.

The two-state behaviour of Mad2 in the cell

The template model of SAC signalling hinges crucially on the existence and interconversion of two conformers of Mad2 (Mapelli and Musacchio, 2007; Luo and Yu, 2008). Using CM2²⁷⁶, we have been able to directly visualize the proposed two-state behaviour of Mad2 within living cells. The fact that the C-Mad2 mAb could only immunoprecipitate Mad2 when bound to interaction partners strongly supports the concept that liganded but not free Mad2 adopts the closed conformation, and our data indicate that this conclusion holds true throughout the cell cycle. As we show here, most of Mad2 was released as a free moiety when Mad1 was depleted, and this free Mad2 existed in the open conformation (as it was not recognized by CM2²⁷⁶). Our data demonstrate that Mad1 is strictly required for Mad2 to adopt the closed conformation *in vivo*. Previous work had already suggested that free Mad2 adopts the open conformation in cells (Luo *et al*, 2004), and this important notion had been incorporated in the template model (De Antoni *et al*, 2005). However, a recent *in vitro* reconstitution study has also suggested that Mad1:C-Mad2 is able to generate unliganded C-Mad2, which then bound Cdc20 with higher efficiency than O-Mad2 (Yang *et al*, 2008). In our study, we did not detect significant levels of unliganded C-Mad2, suggesting that, *in vivo*, the lifetime of this species must be short, if it exists at all. As C-Mad2 is the energetically favoured conformer *in vitro* (Luo *et al*, 2004), this raises the possibility that in the cytoplasm of living cells an as yet unknown mechanism actively promotes the open conformation of Mad2.

Regulation of the Mad1:C-Mad2 complex by p31comet

Considering the central role of the Mad1:C-Mad2 complex in SAC signalling, it is clearly important to understand its regulation in time and space. On the one hand, KTs provide an essential microenvironment for the activity of

the Mad1:C-Mad2 complex, as indicated by the observation that the complex is removed from KTs in a dynein-dependent manner upon SAC silencing (Howell *et al*, 2001) and interference with this removal generates a SAC-dependent arrest (Griffis *et al*, 2007; Gassmann *et al*, 2010). On the other hand, SAC signalling is known to be inhibited by p31comet (Habu *et al*, 2002; Xia *et al*, 2004). Our present study provides further insight into the role of this protein. Immunostaining with CM2²⁷⁶ revealed that the accessibility of C-Mad2 is controlled by p31comet at multiple locations. In particular, our siRNA experiments show that the dimerization interface of C-Mad2 is masked by p31comet not only at NPCs and spindle poles, but also, in part, at KTs. In support of this conclusion, p31comet could be detected at KTs (Habu *et al*, 2002; this study). This raises the question of why a protein implicated in silencing of the SAC associates with the catalytic source of the inhibitory SAC signal already during prometaphase. One possible explanation is that p31comet begins to cap and functionally inactivate individual C-Mad2 proteins as soon as a threshold of bipolar MT attachment to KTs has been reached, perhaps in preparation for subsequent stripping of the Mad1:C-Mad2:p31comet complex from KTs. Alternatively, it would be premature to exclude that p31comet may have not only an inhibitory but also an activating role in SAC signalling, similar to the dual role of securin in the regulation of separase (Hornig *et al*, 2002). Future studies will have to explore these possibilities. In addition, it will be important to better understand the role of posttranslational modifications in the formation and activity of KT-associated complexes (Burke and Stukenberg, 2008; Hewitt *et al*, 2010). Finally, it is noteworthy that the regulation of the p31comet interaction with the Mad1:C-Mad2 complex could not be visualized by analysing bulk populations through biochemical approaches (Supplementary Figures S1D and S5A), suggesting that this regulation concerns only a minor subpopulation of the two proteins (notably the fraction of the complex that localizes at KTs). This further emphasizes the importance of tools such as CM2²⁷⁶ for uncovering local regulation.

The Mad1:C-Mad2 complex is required to both initiate and maintain the SAC

Several predictions of the template model find strong support in the microinjection experiments carried out in our study. The injection of CM2²⁷⁶ abolished SAC activation, thus confirming the template model's prediction that pinpoints the dimerization interface as the active site of C-Mad2 catalysis (De Antoni *et al*, 2005; Kulukian *et al*, 2009; Lad *et al*, 2009; Simonetta *et al*, 2009). One interesting emerging view is that the C-Mad2:Cdc20 complex generated at KTs is not the final inhibitory component of the SAC, but that C-Mad2 acts catalytically—rather than stoichiometrically—to promote Cdc20 binding to BubR1 (Nilsson *et al*, 2008; Kulukian *et al*, 2009). From this perspective, it is interesting that interference with the Mad1:C-Mad2 KT template by injection of CM2²⁷⁶ into cells in which the BubR1:Cdc20 complex was already functional, also caused an abrupt extinction of the SAC. This indicates that the continuous production of new C-Mad2:Cdc20 is required for maintaining a SAC-dependent arrest, even after BubR1 had been loaded onto Cdc20. This in turn suggests that Mad2 is required for SAC maintenance by exerting a BubR1-independent inhibitory action on Cdc20

and/or by continuously replenishing an intrinsically unstable BubR1:Cdc20 complex (Burton and Solomon, 2007; King *et al*, 2007; Nilsson *et al*, 2008).

One interesting hypothesis in the SAC field is that cytoplasmic C-Mad2:Cdc20 can promote its own formation through an autocatalytic loop (De Antoni *et al*, 2005). This idea finds experimental support through *in vitro* experiments (Lad *et al*, 2009; Simonetta *et al*, 2009), but the *in vivo* contribution of a cytosolic branch of Mad2 template activity remains to be tested. From this perspective, it is interesting that microinjection (into nocodazole-arrested cells) of an anti-Mad1 mAb (targeting exclusively the Mad1:C-Mad2 complex) was at least as efficient in causing a SAC override as injection of CM2²⁷⁶ (targeting both KT-associated Mad1:C-Mad2 and cytosolic C-Mad2:Cdc20 complexes). Although these quantitative differences may well reflect a difference in the function-neutralizing potency of the injected antibodies, our observation clearly argues that a C-Mad2:Cdc20 autocatalytic loop is not in itself sufficient to maintain a SAC arrest.

KT-dependent control of mitotic timing

Careful live-cell studies in siRNA-treated human cells have led to the proposal that anaphase onset depends on a mitotic timer that involves Mad2 and BubR1, but not Mad1 (Meraldi *et al*, 2004). Genetic analyses in *Drosophila* are consistent with such a timer model, although the contributions of Mad2 and BubR1 to mitotic timing in flies appear to be minor and, remarkably, the SAC is not strictly required for undisturbed mitoses in this organism (Buffin *et al*, 2007; Rahmani *et al*, 2009; Emre *et al*, 2011). In the present study, injection of cells with antibodies directed at either C-Mad2 or Mad1 triggered a striking mitotic acceleration. These results cannot readily be reconciled with the proposal that Mad2 (together with BubR1) is part of a 'cytoplasmic mitotic timer', which works independently of the SAC (Meraldi *et al*, 2004). Although we cannot formally exclude that functional neutralization by mAb microinjection triggers a different (and even stronger) phenotype than the reduction of the corresponding proteins by siRNA-mediated depletion, we suspect that the reported differences in the phenotypes observed after siRNA-mediated depletion of Mad1 or Mad2 (Meraldi *et al*, 2004; our unpublished data) reflect differences in the functionality of residual protein levels. In particular, Mad2 depletion simultaneously affects both the KT-associated Mad1-bound Mad2 population (C-Mad2) and the cytoplasmic pool of Mad2 that awaits catalytic conversion (O-Mad2). Hence, even partial reduction of Mad2 readily causes acceleration of traverse through mitosis (Hubner *et al*, 2010). Conversely, Mad1 depletion affects exclusively the catalyst (the Mad1:C-Mad2 complex) and, therefore, minor amounts of residual Mad1 might be sufficient to partly support the checkpoint, thus preventing mitotic acceleration. A recent study has also implicated the SAC kinase Mps1 in the regulation of mitotic timing, suggesting that Mps1 is also required during interphase to form an anaphase inhibitor in a KT-independent manner (Maciejowski *et al*, 2010). However, as our present data demonstrate that functional abrogation of Mad1:C-Mad2 also accelerates mitotic progression, we would predict that complete neutralization of any SAC component, including Mps1, will translate into faster progression through mitosis. Most importantly, the consequences of Mps1 inhibition were

indistinguishable, regardless of whether the kinase was inhibited already during interphase or only during mitosis. Thus, interphase activity of Mps1 does not significantly contribute to the timing of mitotic progression. Collectively, our results on the role of Mad1:C-Mad2 and Mps1 should prompt a reconsideration of the distinction between SAC and mitotic timer.

Materials and methods

Generation of CM2²⁷⁶

CM2²⁷⁶ (clone 107-276) has been described previously (Chan *et al*, 2009). Briefly, CM2²⁷⁶ (IgG1k) was generated against GST-tagged full-length human Mad2 by repeated subcutaneous injection of 100 µg of antigen into Balb/c mice using aluminium hydroxide as an adjuvant. Spleen cells were fused with PAIB₃Ag81 mouse myeloma cells. Supernatant screening was performed by ELISA.

Competition assays

For GST pull-down experiments in Figure 1D–F and Supplementary Figure S1E and F, a complex between GST–Cdc20^{111–138} and His-tagged Mad2^{WT} was generated on glutathione-sepharose 4B beads (GE Healthcare) as described before (Mapelli *et al*, 2007). Competition in binding to C-Mad2 was assessed by adding CM2²⁷⁶ to the beads at 0.2 µM together with p31comet at 0.6, 3.6 or 9 µM (Figure 1D). The corresponding negative control (Supplementary Figure S1E) was performed by replacing p31comet with equal concentrations of hSpindly^{1–444}. In Figure 1E, Mad2^{ΔC} was added to the beads at a concentration of 0.35 µM together with CM2²⁷⁶ at concentrations of 0.15, 0.45 or 1.35 µM. The corresponding negative control (Supplementary Figure S1F) was performed by replacing CM2²⁷⁶ with equal concentrations of Mad1 mAb (117–468). For the experiment in Figure 1F, Mad2^{ΔC} and CM2²⁷⁶ were used at concentrations of 0.35 and 0.9 µM, respectively.

SEC followed by IP

Synchronized HeLaS3 cells were lysed as described in the Supplementary data. A measure of 1 ml of clarified lysate containing 10 mg of total protein extract was loaded on a Superdex 200 16/60 column (GE) using an Äkta Explorer FPLC system (Amersham). An isocratic elution was performed at 4°C applying a constant flow of 1.1 ml/min of 20 mM Tris–HCl, pH 7.4, 150 mM NaCl. Fractions were collected every 4 ml; protease and phosphatase inhibitors were added to each fraction as to the lysis buffer. Each fraction was subdivided in a 1.3-ml part and a 2.7-ml part, destined to trichloroacetic acid (TCA) precipitation and IP, respectively. IP was performed for 2 h at 4°C using 20 µl of solid protein A beads (Bio-Rad), previously coupled and cross-linked to the CM2²⁷⁶ at 1 µg/µl. Beads were washed three times with 0.5 ml of elution buffer and resuspended in 30 µl of Laemmli buffer. TCA-precipitated protein pellets were resuspended in 80 µl of Laemmli buffer. A total of 10 µl was further analysed by SDS–PAGE and WB.

Antibody microinjection

Antibody injection was performed using Femtotips II capillaries operated on a FemtoJet microinjector (Eppendorf). Antibodies were injected at a needle concentration of 1–2 mg/ml in PBS in the presence of 1.66 mg/ml Texas Red dextran or Alexa Fluor 488 dextran (both 10,000 MW; Invitrogen). Each injection was performed for 0.2–0.3 s with injection and compensation pressures of 15 hPa. To assess the SAC functionality and effects on mitotic timing upon mAb microinjection, HeLaS3 H2B–GFP (Sillje *et al*, 2006) cells or HeLa Kyoto H2B–mCherry (Neumann *et al*, 2010) were seeded on 35 mm µ-Dishes or coverslips and synchronized as described in the corresponding Figure legends 6–8. Different mAbs were injected at different grid coordinates within the same plate or on different coverslips and phenotypes were analysed by live-cell imaging or immunofluorescence microscopy.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

Acknowledgements

We are especially grateful to A Musacchio for reagents, insight on the project and comments on the manuscript. We also thank J Ellenberg, AA Hyman, I Poser, NS Gray, A Uldschmid and YW Chan for reagents and A Wehner and E Nigg for technical help. We thank all past and present members of our laboratory for insightful discussion. This work was supported by the Max Planck Society, the University of Basel and the Experimental Network for Functional Integration (ENFIN) Contract LSHG-CT-2005-518254 funded by the European Commission within its FP6 Programme.

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LLF was partly supported by the International Max Planck Research School for Molecular and Cellular Life Sciences.

Author contributions: LLF, EAN and AS conceived and designed the experiments. LLF and MK carried out the experiments. LLF and AS analysed the data. LLF, EAN and AS wrote the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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