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Supplementary Discussion

Section A. Apparent dissociation constant

The crystal structure of MCC (Extended data Figure 1b) shows that each of the three interacting subunits (CDC20, MAD2, and BUBR1) has binding interfaces for the other two. One can therefore identify three different dissociation constants, one for each binary interaction, i.e. MAD2:CDC20, MAD2:BUBR1, and CDC20:BUBR1. We designate these dissociation constants as K_d(MC), K_d(MB), and K_d(CB). K_d(MC) is 150 nM (see Figure 1c). At the concentrations of MAD2 and BUBR1 used in our assays, we did not observe binding of MAD2 with BUBR1 in the absence of CDC20 (Extended data Figure 2d), indicating that K_d(MB) is high (i.e. the affinity is low). As clarified in the text, we could not measure K_d(CB) directly. When MAD2, CDC20, and BUBR1 are combined, the overall affinity increases because the multiple interfaces involved in the binary interactions identified above can combine in a single complex. In the case of Sensor 1, we define as apparent K_d the concentration of MAD2 responsible for half-maximal saturation of the Sensor's FRET signal at saturating concentrations of BUBR1. In the case of Sensor 2, the same definition is adopted, with the difference that the signal is now measured in presence of saturating concentrations of CDC20 instead of BUBR1. As expected, the two apparent K_ds are identical, as they reflect the same physical interactions of the MCC subunits when they are all present.

Section B. Cellular concentrations of checkpoint proteins

The following cellular concentration ranges (only lowest and highest values are indicated) have been reported for the indicated SAC subunits in mammalian cell culture systems:

-BUB1: 100 nM (reference 1)

-BUBR1: 90-130 nM (references 2-4)

-CDC20: 100-285 nM (references 2,4)

-MAD1: 20 nM or ¼ of MAD2 (references 5,6)

-MAD2: 120-400 nM (references 2-7)

In extracts of *Xenopus laevis*, Cdc20, Mad1, and Mad2 were found at 10 nM, 50 nM, and 200 nM, respectively[8]. Concentration estimates for *Schizosaccharomyces pombe* SAC proteins have also been published and found to be in a range similar to that observed in higher eukaryotes[9].

Section C. The MAD2-template model

The MAD1:C-MAD2 complex stands out as a putative catalyst for conversion of O-MAD2[10-12]. MAD1, which is otherwise unrelated to CDC20, contains a MIM related to that of CDC20[13,14] (Extended data Figure 1d). It binds O-MAD2, acting as its receptor at kinetochores, and makes it adopt the C-MAD2 conformation. Contrarily to MCC, which is actively disassembled to allow APC/C re-activation and mitotic exit, the MAD1:C-MAD2 complex is a stable 2:2 tetramer already present when MCC assembly begins in early mitosis[14-16].

C-MAD2 in the MAD1:C-MAD2 complex activates O-MAD2 via a mechanism of conformational dimerization[17,18] (Extended data Figure 1e). Mutations impairing O-MAD2:C-MAD2 dimerization, or depletion of MAD1, prevent MCC accumulation and SAC signalling, suggesting that the MAD1:C-MAD2 complex promotes binding of MAD2 to CDC20[17,19,20]. Furthermore, the outline of this interaction seems to suggest that C-MAD2 in the MAD1:C-MAD2 complex is a structural template that stimulates, by binding to it, the conversion of an O-MAD2 substrate to a C-MAD2 copy bound to CDC20. This hypothesis is named the MAD2 template model[12,17]. It has until now remained unproven, because previous studies identified only modest effects of MAD1:C-MAD2 on the kinetics of MAD2:CDC20 accumulation, insufficient for rapid accumulation of MCC in living cells[10,11]. Here, we provide evidence consistent with major assumptions of the model.

Section D. Choice of catalysts' concentrations

As shown in Figure 2b, catalytic MCC production is observed also at very low concentrations of catalysts, and the catalytic reaction responds robustly to lower MPS1 concentrations (Extended data figure 8a). In many experiments in Figures 3-4 and Extended data figure 8, however, we used equimolar concentrations of catalysts and reagents (~100 nM), which essentially enforces a turnover of a single event per catalyst. Very high catalyst concentration is the most unfavorable condition for detecting a perturbation of the catalytic machinery (e.g. at this concentration, a 10-fold reduction of MPS1 concentration has very marginal effects on reaction rates, see Extended data figure 8a), and we reasoned that any strong effect on catalytic rates observed in a perturbation experiment under this regime should be considered penetrant and striking.

Section E. Previous evidence of interactions of MPS1, BUB1:BUB3, and MAD1:C-MAD2

An interaction between Mad1:C-Mad2 and Bub1:Bud3 was originally identified in *S. cerevisiae*[21],

and later in *C. elegans*[22]. This interaction mediates kinetochore association of Mad1:C-Mad2 and requires the phosphorylation by Mps1 of a region within Bub1 (reference 23). Whether the MAD1:C-MAD2 interaction with BUB1:BUB3 in humans is mediated by the same mechanism is unknown. We note that we can reconstitute catalysis with separate pre-incubations (in presence of ATP) of MAD1:C-MAD2 with MPS1 and BUB1:BUB3 with itself, suggesting that MPS1 does not need to phosphorylate BUB1:BUB3 for catalysis to occur (Extended data Figure 6c). For similar reasons, the previously reported phosphorylation of MAD1 by BUB1 (reference 24) may not be relevant in our system. The interaction of MAD1 with BUB1, or more generally the functionality of MAD1 in the SAC, requires the C-terminal RWD region of MAD1 (Extended data Figure 1d and references 21,25-27).

In other model systems, including human cultured cells and *S. pombe*, the role of Mad1 has been shown to extend beyond its function as placeholder for C-Mad2 (references 26,27). MPS1 has a clear role in the localization of BUB1:BUB3 as well as of MAD1:C-MAD2 (references 28-43). BUB1:BUB3, on the other hand, has been implicated in the recruitment of MAD1:C-MAD2 (references 25,32,33,44,45), together with the ROD-ZWILCH-ZW10 (RZZ) complex[46].

Besides contributing to MAD1:C-MAD2 kinetochore localization, MPS1 has been shown to be continuously required for the template function of the MAD1:C-MAD2 complex, in particular by favoring an interaction with O-MAD2 (reference 29). MPS1 has been shown to phosphorylate MAD1 and C-MAD2 (references 47,48). However, the interaction of O-MAD2 with MAD1:C-MAD2 does not require MPS1 activity[11,17,49,50], suggesting that phosphorylation regulates a different aspect of the interaction of O-MAD2 with C-MAD2.

Section F. Role of catalysis in MCC dynamics

Our previous work identified MAD1:C-MAD2 as the receptor for the recruitment of O-MAD2 to kinetochores required for its binding with CDC20 (reference 17). This, and the realization that the previously reported dimerization of MAD2 (reference 51) engages structurally different O-MAD2 and C-MAD2 conformers[17,18], led to the formulation of the MAD2 template model[17] (discussed in Section C). Implicit in the template model is the idea that the interaction of MAD2 conformers, shown to be required for checkpoint function, lowers the energy barrier dividing MAD2 conformers and accelerates the conversion. Previously, we used a simple fluorescence-based assay designed to monitor binding of O-MAD2 with an immobilized synthetic peptide encompassing the CDC20 MIM. With this tool, we showed modest (~8-fold) acceleration of the rate of CDC20:C-MAD2 complex formation when adding MAD1:C-MAD2. This rate

enhancement was clearly insufficient for establishing a robust checkpoint signal[11]. Thus, in our previous studies we were not able to reconstitute strong catalytic activation of the mitotic checkpoint *in vitro*, likely because we lacked the kinase activity that we now show to be essential and because we used a MAD1:MAD2 construct containing MAD1$^{485\text{-}718}$, which we show here to be partly impaired as a catalyst.

In a study by Kulukian and coworkers[10], purified chromosomes were shown to impart a modest increase to the rate of CDC20:C-MAD2 complex formation, and MAD1 was identified as one of the chromosomal components required for robust APC/C inhibition by recombinant MCC subunits. However, the minimal requirements for catalytic activation of checkpoint signaling at kinetochores had remained unclear and uncharacterized in the work of Kulukian and co-workers[10].

Here, we have overcome the fundamental limitations of both of these important previous studies by identifying at least some of the crucial steps of catalytic activation of MCC formation. The >100-fold acceleration of the rate of CDC20:C-MAD2 assembly at near physiological concentrations reported in our analysis can explain the rapid re-activation of checkpoint signaling observed in living cells[52,53], although the role of kinetochores requires further investigation.

MAD2 and BUBR1 inhibit CDC20-mediated activation of APC/C synergistically[2,4], suggesting that the pairwise interactions of CDC20, MAD2 and BUBR1 observed in the MCC^{core} trimer (Extended data Figure 1b) produce considerable augmentation of overall binding affinity[54]. Kulukian and co-workers and later Han and co-workers[10,55] provided an alternative interpretation of the synergistic effects of MAD2 and BUBR1. These authors proposed that MAD2 operates catalytically to promote binding of CDC20 to BUBR1:BUB3[10,55], and identified the CDC20:BUBR1 complex as the *bona fide* inhibitor of the APC/C[10,55]. This idea is inconsistent with our observations. MCC^{core} is a cooperative assembly, as already suggested by its crystal structure[54]. Addition of unlabeled CDC20 to fluorescent MAD2 and BUBR1 drives an extremely tight association of MAD2 and BUBR1. Thus, MAD2 is a tightly bound subunit of the resulting MCC complex, whose extraction from the complex, at physiologic concentrations, requires an energy intake. This energy intake likely comes from ATP hydrolysis by a AAA+ ATPase named PCH2^{TRIP13} (reviewed in [56,57]).

Section G. Why is the <u>normalized</u> rate of MCC formation with Sensor 2 inversely proportional to concentration of BUBR1:BUB3?

As already clarified in the main text, binding of MAD2^{TAMRA} with dark CDC20 is required for the

interaction of MAD2^{TAMRA} with ^{CFP}BUBR1. The latter gives rise to the FRET signal of MCC Sensor 2. Binding of MAD2^{TAMRA} with dark CDC20 is not only required, but also rate limiting for MCC FRET Sensor 2 accumulation. CDC20:MAD2^{TAMRA} and ^{CFP}BUBR1 bind with very high affinity, and ^{CFP}BUBR1 is essentially under saturating conditions at its concentrations in our assay (because its concentration is usually well above the effective dissociation constant). Therefore, any new CDC20:MAD2^{TAMRA} that is formed binds immediately and with very high affinity to ^{CFP}BUBR1. The lower the ^{CFP}BUBR1 concentration, the lower CDC20:MAD2^{TAMRA} needs to be formed to bind with ^{CFP}BUBR1. If we normalize FRET signal at any time *t* to the maximum signal at saturation, a reaction running with lower ^{CFP}BUBR1 concentrations will appear to reach saturation faster than a reaction at higher ^{CFP}BUBR1 concentration, as the latter will require a higher overall amount of CDC20:MAD2^{TAMRA} to be formed before incorporation of all BUBR1 in MCC is achieved. In Extended data Figure 4c, the halftimes ($t_{1/2}$) for MCC Sensor 2 accumulation were respectively 107, 160 and 215 minutes for ^{CFP}BUBR1 concentrations of 10 nM, 25 nM, and 100 nM. These halftimes reflect the accumulation of MCC equivalents required for MCC to form. Of course, due to the fact that forming CDC20:C-MAD2^{TAMRA} is rate limiting, and that BUBR1 is always at saturating concentrations in these experiments, the absolute levels of core MCC formed at any given time *t* at different concentrations of BUBR1:BUB3 are identical.

Supplementary Discussion References

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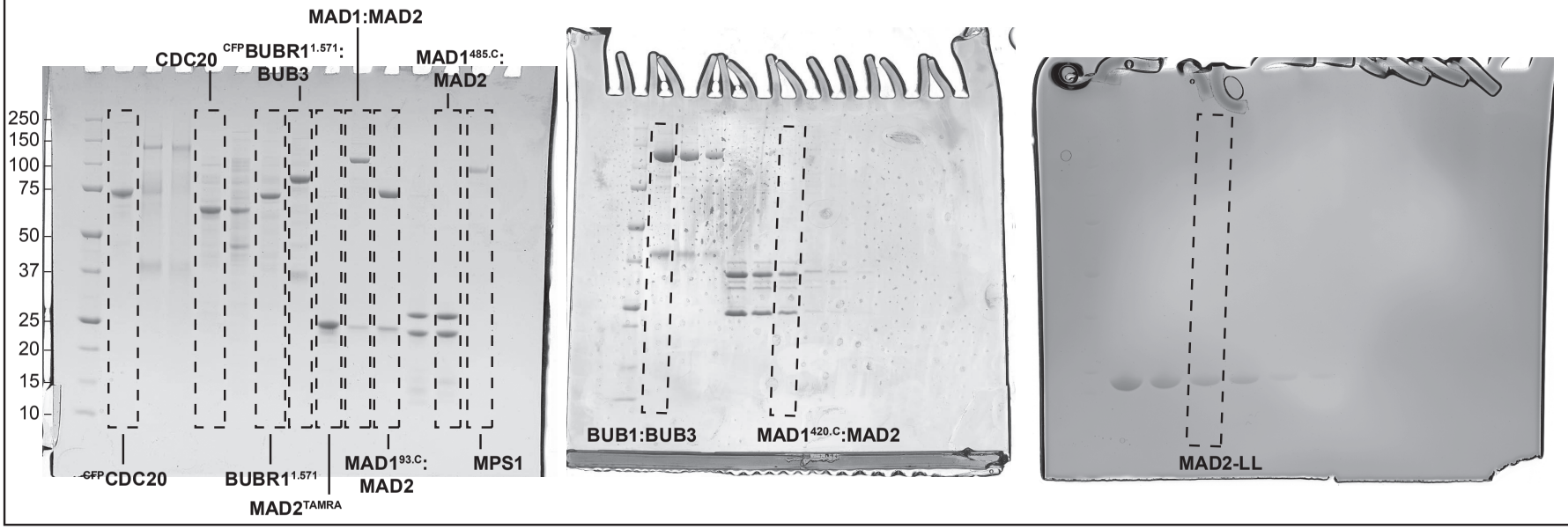
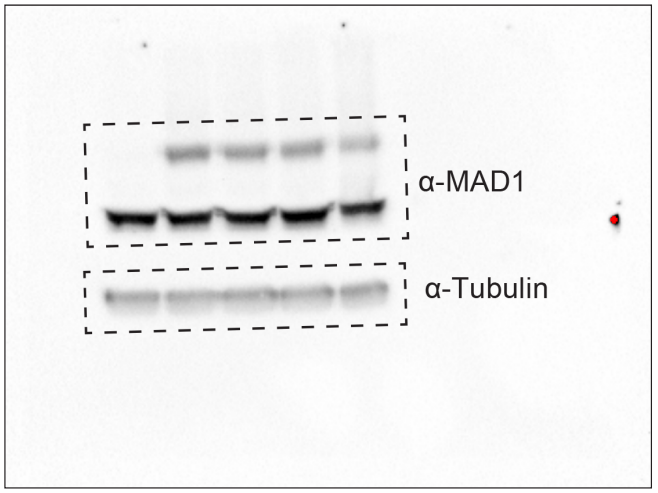
Coomassie stained gels Extended data Figure 2a



Western Blots Extended data Figure 7c



Western Blots Extended data Figure 8d