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A tripartite mechanism catalyzes Mad2–Cdc20 assembly at unattached kinetochores

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Abstract

During cell division, kinetochores couple chromosomes to spindle microtubules. To protect against chromosome gain or loss, kinetochores lacking microtubule attachment locally catalyze association of the checkpoint proteins Cdc20 and Mad2, which is the key event in the formation of a diffusible checkpoint complex that prevents mitotic exit. We elucidated the mechanism of kinetochore-catalyzed Mad2–Cdc20 assembly with a probe that specifically monitors this assembly reaction at kinetochores in living cells. Catalysis occurred through a tripartite mechanism that included localized delivery of Mad2 and Cdc20 substrates and two phospho-dependent interactions that geometrically constrained their positions and primed Cdc20 for interaction with Mad2. These results reveal how unattached kinetochores create a signal that ensures genome integrity during cell division.

One Sentence Summary:

A tripartite mechanism explains how the kinetic barrier to mitotic checkpoint complex assembly is overcome at kinetochores.

During cell division, the centromere regions of replicated chromosomes assemble kinetochores, mechanical interfaces that couple sister chromatids to spindle microtubules (1). Kinetochores also serve as signaling hubs that monitor their own attachment status; when unattached, they delay anaphase onset by producing an inhibitor of the anaphase-

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Supplementary Materials:
Materials and Methods
Figures S1–S8
Tables S1–S5
References (22–32)

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promoting complex/cyclosome (APC/C), the E3 ubiquitin ligase that promotes exit from mitosis (2). Prior work has shown that the essential role of kinetochores in APC/C inhibitor production is to catalyze formation of a complex between the checkpoint proteins Mad2 and Cdc20 (3). Once formed, the Mad2–Cdc20 complex rapidly binds Mad3 (BubR1) and Bub3 in the cytosol to form the mitotic checkpoint complex that inhibits the APC/C. Central to complex formation is the ability of Mad2 to adopt two conformational states: an open, free form and a closed ligand-bound form (4, 5). The Mad2 ligands in checkpoint signaling are Mad1, a dimeric coiled-coil protein with a folded C-terminal domain, and Cdc20 (4, 5). The Mad1–Mad2 complex is present throughout the cell cycle whereas Mad2 assembly onto Cdc20 is kinetically disfavored and requires catalysis by unattached kinetochores (Fig. 1A; (6, 7)). In the current model of checkpoint signaling, a stable kinetochore-anchored complex of Mad1 with closed Mad2 recruits an open conformer of Mad2 from the cytoplasm via asymmetric dimerization; this open Mad2 is subsequently linked to Cdc20 (Fig. 1A; (4, 5)). How kinetochores overcome the kinetic barrier to Mad2–Cdc20 association is not understood. Reconstitutions have highlighted a role for Mad1 phosphorylation (6, 8) but it is unclear how this phosphorylation acts, whether it is sufficient for catalysis in vivo, and why catalysis occurs specifically at kinetochores.

The presence of two kinetochore-localized pools of Mad2 (9) has prevented selective monitoring of the cycling pool that becomes linked to Cdc20, limiting efforts to unravel the steps involved in Cdc20-Mad2 assembly at kinetochores. In an effort to in situ tag MAD-2 with green fluorescent protein (GFP) in *C. elegans* embryos, we fortuitously generated a C-terminal MAD-2::GFP fusion that enables specific visualization of the cycling pool of Mad2 at kinetochores in living cells. MAD-2::GFP localization to unattached kinetochores was compromised when it was the sole source of MAD-2 (Fig. 1B; and Fig. S1A). Embryos expressing only MAD-2::GFP were defective in checkpoint signaling (10), which initially suggested loss of MAD-2 function. However, MAD-2::GFP localized to kinetochores when expressed together with untagged MAD-2 (Fig. 1B, and Fig. S1B), suggesting that MAD-2::GFP can be recruited to kinetochores through dimerization with untagged MAD-2 bound to MAD-1. Consistent with this model, engineering MAD-1 to selectively disrupt MAD-2 binding or disrupting the dimerization interface on MAD-2::GFP both eliminated MAD-2::GFP kinetochore localization (Fig. 1C and D, Fig. S1C and Fig. S2). Thus MAD-2::GFP, when expressed alongside untagged MAD-2, selectively monitors the MAD-2 that cycles through kinetochores during checkpoint signaling (Fig. S3).

The cycling pool of Mad2 localizes to kinetochores through asymmetric dimerization with Mad2 stably bound to Mad1, and then undergoes conformational conversion to a closed Cdc20-bound state (3, 11). To test which of these events is required to observe kinetochore localized MAD-2::GFP, we generated a locked-open mutant that exhibits normal dimerization but cannot undergo open-to-closed conversion (Fig. 2A, and Fig. S2A and B). Locked-open MAD-2::GFP was not detected at kinetochores, indicating that stable binding of cycling MAD-2 at kinetochores depends not only dimerization with untagged MAD-2, but also conversion of MAD-2 to its CDC-20–bound closed form. Supporting this model, both CDC-20's MAD-2-interaction motif (10) and the ABBA motif of BUB-1 that recruits CDC-20 to kinetochores (12, 13) were required for MAD-2::GFP kinetochore localization (Fig. 2B and C, and Fig. S4A). These results indicate that the MAD-2::GFP probe reveals

MAD-2–CDC-20 complex formation at kinetochores. Consistent with this idea, locked-open MAD-2::GFP dominantly inhibited checkpoint signaling in the presence of untagged MAD-2 (Fig. S2C).

If MAD-2::GFP monitors the rate-limiting step of mitotic checkpoint complex assembly–formation of the MAD-2–CDC-20 complex at kinetochores–MAD-2::GFP localization should be unaffected by removal of MAD-3 (Fig. S4B and C). In fact, MAD-3 depletion increased MAD-2::GFP signal at kinetochores (Fig. 2D), indicating that capture by MAD-3 may aid release of MAD-2::GFP–CDC-20 complexes from kinetochores.

Adjacent to its Cdc20-binding ABBA motif, Bub1 contains a conserved motif (CM1) that, when phosphorylated, mediates an interaction with the Mad1 C-terminus ((8, 14, 15); Fig. S5A). Although Mad1–Mad2 kinetochore localization depends on phosphorylated BUB-1 CM1 in some species (14–16), its importance in the checkpoint signaling is independent of Mad1–Mad2 localization (16), suggesting a second, more conserved function. In *C. elegans*, mutating BUB-1 CM1 had no effect on MAD-1 kinetochore localization and only a mild effect on CDC-20 localization but eliminated the MAD-2::GFP signal at kinetochores and fully inhibited checkpoint signaling (Fig. 3A to C, and Fig. S5B to D). Consistent with phosphorylation being key to CM1 function in MAD-2–CDC-20 assembly, mutation of a single conserved threonine (Thr407) in CM1 eliminated MAD-2::GFP signal at kinetochores and inhibited checkpoint signaling (Fig. S5E and F). Phosphorylated CM1 interacts with the Mad1 C-terminus (8, 15) and deletion of the MAD-1 C-terminus also eliminated MAD-2–CDC-20 assembly at kinetochores without perturbing MAD-1 localization or MAD-1–MAD-2 complex formation (Fig. S6). Thus, delivering MAD-2 and CDC-20 to kinetochores, via their respective docking sites on the MAD-1–MAD-2 complex and BUB-1, appears insufficient to catalyze complex formation; instead phosphorylation of the BUB-1 CM1 motif and its subsequent interaction with the MAD-1 C-terminus are also required. Consistent with the idea that physical proximity of BUB-1's MAD-1 and CDC-20 binding motifs are critical to mediate MAD-2–CDC-20 assembly, *bub-1* alleles with separate mutations in CM1 and ABBA failed to complement one another (Fig. 3D).

The above data highlight a critical role for the BUB-1 ABBA and CM1 motifs in recruiting and positioning CDC-20 for assembly with MAD-2. However, they do not address why MAD-2–CDC-20 association is disfavored in the cytoplasm, and how kinetochores overcome this barrier. Phosphorylation of the Mad1 C-terminus facilitates interaction with the N-terminal region of Cdc20 and promotes Mad2–Cdc20 complex formation in vitro (8). In humans Mad1 is phosphorylated by Mps1, but *C. elegans* lacks Mps1, and its checkpoint function is provided by Polo-like kinase 1 (PLK-1) (17). Phosphorylation by PLK-1 of the MAD-1 C-terminus promoted its interaction with the CDC-20 N-terminus in vitro (Fig. 4A, and Fig. S7A and B). This interaction was not required to recruit CDC-20 to kinetochores, as CDC-20 localized normally in the absence of the MAD-1 C-terminus (Fig. 4B). In solution, the motif in the Cdc20 N-terminus that interacts with Mad2 is masked, potentially by an interaction with its C-terminal WD40 domain (Fig. S7C; (18, 19)). By interacting with its N-terminus, phosphorylated Mad1 may expose Cdc20's Mad2 interaction motif and, in conjunction with the local concentration and positioning mechanisms described above, drive its assembly onto Mad2 at kinetochores. This idea was tested using MAD-1 mutants that

prevent its phosphorylation-stimulated interaction with the CDC-20 N-terminus; three residues on the MAD-1 RWD domain were targeted for this purpose (Fig. 4C, and Fig. S7A and 7D (20)). The MAD-1 RWD mutant did not exhibit phosphorylation-dependent binding to the CDC-20 N-terminus in vitro; when introduced in vivo, it eliminated formation of the MAD-2::CDC-20 complex at kinetochores and was deficient in checkpoint signaling (Fig. 4D, and Fig. S7E to G). One of the critical phosphorylation sites that mediates interaction with Cdc20 in human Mad1 (Thr716) (6, 8) is not conserved outside of vertebrates (Fig. S8A). Analysis of 6 putative phosphosites in the *C. elegans* MAD-1 C-terminus indicated that one conserved residue (Thr653) was important for MAD-2–CDC-20 association at kinetochores and for checkpoint signaling (Fig. S7F and G, and Fig. S8). These data support a model in which phosphorylation of Mad1 at kinetochores promotes an interaction with Cdc20 that exposes its Mad2 interaction motif (6, 8, 21).

Visualization of kinetochore-localized Mad2–Cdc20 assembly, together with component engineering, reveals how the spindle checkpoint signal is generated at unattached kinetochores (Fig. 4E). Localized signal generation involves three steps: 1) delivery of Cdc20 (by the Bub1 ABBA motif) and Mad2 (through dimerization with the Mad1–Mad2 template); 2) positioning of Cdc20 by interaction of the Bub1 CM1 motif with the Mad1 C-terminus; 3) unfurling of the Cdc20 N-terminus by interaction with phosphorylated Mad1, exposing its Mad2 interaction motif. The requisite multipartite interaction network explains the specific assembly of Mad2–Cdc20 signaling complexes at unattached kinetochores. Within this network, Bub1 plays a key “matchmaker” role, mediating Cdc20 recruitment (via the ABBA motif) and positioning (via the CM1 motif interacting with Mad1) at unattached kinetochores. Catalysis additionally requires phosphorylation of Mad1, which likely serves to unfurl Cdc20 and expose its Mad2 interaction motif. Thus, the simultaneous interaction of Cdc20 with Bub1 and Mad1 is critical for overcoming the kinetic barrier to its association with Mad2. Our conclusions are in broad agreement with those from a biochemical reconstitution of the spindle checkpoint using purified components (21). These two studies address the long-standing question of how kinetochores direct localized production of signaling complexes that act as critical guardians of the genome during cell division.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References and Notes:

1. Musacchio A, Desai A, A molecular view of kinetochore assembly and function. Biology (Basel) 6, (2017).
2. Barford D, Structural interconversions of the anaphase-promoting complex/cyclosome (APC/C) regulate cell cycle transitions. Curr Opin Struct Biol 61, 86–97 (2019). [PubMed: 31864160]
3. Corbett KD, Molecular mechanisms of spindle assembly checkpoint activation and silencing. Prog Mol Subcell Biol 56, 429–455 (2017). [PubMed: 28840248]
4. Luo X, Yu H, Protein metamorphosis: the two-state behavior of Mad2. Structure 16, 1616–1625 (2008). [PubMed: 19000814]
5. Musacchio A, The molecular biology of spindle assembly checkpoint signaling dynamics. Curr Biol 25, R1002–1018 (2015). [PubMed: 26485365]
6. Faesen AC et al., Basis of catalytic assembly of the mitotic checkpoint complex. Nature 542, 498–502 (2017). [PubMed: 28102834]
7. Fang G, Checkpoint protein BubR1 acts synergistically with Mad2 to inhibit anaphase-promoting complex. Mol Biol Cell 13, 755–766 (2002). [PubMed: 11907259]
8. Ji Z, Gao H, Jia L, Li B, Yu H, A sequential multi-target Mps1 phosphorylation cascade promotes spindle checkpoint signaling. Elife 6, (2017).
9. Shah JV et al., Dynamics of centromere and kinetochore proteins; implications for checkpoint signaling and silencing. Curr Biol 14, 942–952 (2004). [PubMed: 15182667]
10. Lara-Gonzalez P et al., The G2-to-M transition is ensured by a dual mechanism that protects cyclin B from degradation by Cdc20-activated APC/C. Dev Cell 51, 313–325 e310 (2019). [PubMed: 31588029]
11. Zhang G, Nilsson J, The closed form of Mad2 is bound to Mad1 and Cdc20 at unattached kinetochores. Cell Cycle 17, 1087–1091 (2018). [PubMed: 29895228]
12. Di Fiore B et al., The ABBA motif binds APC/C activators and is shared by APC/C substrates and regulators. Dev Cell 32, 358–372 (2015). [PubMed: 25669885]
13. Kim T et al., Kinetochores accelerate or delay APC/C activation by directing Cdc20 to opposing fates. Genes Dev 31, 1089–1094 (2017). [PubMed: 28698300]
14. Klebig C, Korinth D, Meraldi P, Bub1 regulates chromosome segregation in a kinetochore-independent manner. J Cell Biol 185, 841–858 (2009). [PubMed: 19487456]
15. London N, Biggins S, Mad1 kinetochore recruitment by Mps1-mediated phosphorylation of Bub1 signals the spindle checkpoint. Genes Dev 28, 140–152 (2014). [PubMed: 24402315]
16. Heinrich S et al., Mad1 contribution to spindle assembly checkpoint signalling goes beyond presenting Mad2 at kinetochores. EMBO Rep 15, 291–298 (2014). [PubMed: 24477934]
17. Espeut J et al., Natural loss of Mps1 kinase in nematodes uncovers a role for Polo-like Kinase 1 in spindle checkpoint initiation. Cell Rep 12, 58–65 (2015). [PubMed: 26119738]
18. Zhang Y, Lees E, Identification of an overlapping binding domain on Cdc20 for Mad2 and anaphase-promoting complex: model for spindle checkpoint regulation. Mol Cell Biol 21, 5190–5199 (2001). [PubMed: 11438673]
19. Han JS et al., Catalytic assembly of the mitotic checkpoint inhibitor BubR1–Cdc20 by a Mad2-induced functional switch in Cdc20. Mol Cell 51, 92–104 (2013). [PubMed: 23791783]
20. Kim S, Sun H, Tomchick DR, Yu H, Luo X, Structure of human Mad1 C-terminal domain reveals its involvement in kinetochore targeting. Proc Natl Acad Sci U S A 109, 6549–6554 (2012). [PubMed: 22493223]
21. Piano V et al., Cdc20 assists its catalytic incorporation in the mitotic checkpoint complex as substrate of a non-canonical chaperone. Co-submitted to Science.
22. Frokjaer-Jensen C et al., Single-copy insertion of transgenes in Caenorhabditis elegans. Nature genetics 40, 1375–1383 (2008). [PubMed: 18953339]
23. Moyle MW et al., A Bub1-Mad1 interaction targets the Mad1–Mad2 complex to unattached kinetochores to initiate the spindle checkpoint. J Cell Biol 204, 647–657 (2014). [PubMed: 24567362]

24. Hattersley N et al., Employing the one-cell C. elegans embryo to study cell division processes. Methods Cell Biol 144, 185–231 (2018). [PubMed: 29804670]

25. Dickinson DJ, Pani AM, Heppert JK, Higgins CD, Goldstein B, Streamlined genome engineering with a self-excising drug selection cassette. Genetics 200, 1035–1049 (2015). [PubMed: 26044593]

26. Wang S et al., A toolkit for GFP-mediated tissue-specific protein degradation in C. elegans. Development 144, 2694–2701 (2017). [PubMed: 28619826]

27. Mapelli M, Massimiliano L, Santaguida S, Musacchio A, The Mad2 conformational dimer: structure and implications for the spindle assembly checkpoint. Cell 131, 730–743 (2007). [PubMed: 18022367]

28. De Antoni A et al., The Mad1/Mad2 complex as a template for Mad2 activation in the spindle assembly checkpoint. Curr Biol 15, 214–225 (2005). [PubMed: 15694304]

29. Essex A, Dammermann A, Lewellyn L, Oegema K, Desai A, Systematic analysis in Caenorhabditis elegans reveals that the spindle checkpoint is composed of two largely independent branches. Mol Biol Cell 20, 1252–1267 (2009). [PubMed: 19109417]

30. Brady DM, Hardwick KG, Complex formation between Mad1p, Bub1p and Bub3p is crucial for spindle checkpoint function. Curr Biol 10, 675–678 (2000). [PubMed: 10837255]

31. Zhang G et al., Bub1 positions Mad1 close to KNL1 MELT repeats to promote checkpoint signalling. Nat Commun 8, 15822 (2017). [PubMed: 28604727]

32. Ashkenazy H et al., ConSurf 2016: an improved methodology to estimate and visualize evolutionary conservation in macromolecules. Nucleic Acids Res 44, W344–350 (2016). [PubMed: 27166375]

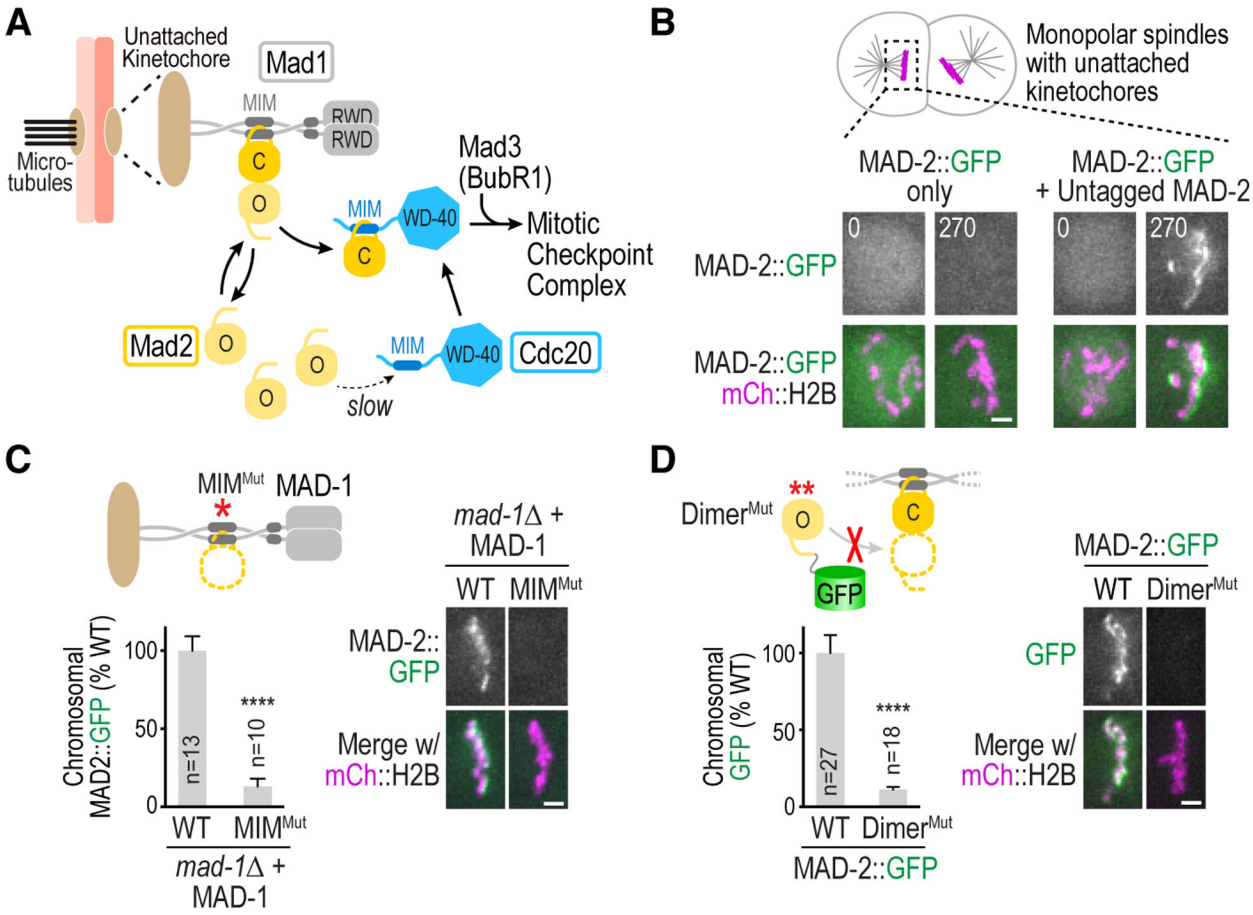

Figure 1: A probe that selectively visualizes the cycling pool of Mad2 at kinetochores.

(A) Schematic of spindle checkpoint signaling. "O" and "C" indicate open and closed Mad2 conformers, respectively. See text for details. **(B)** Localization of MAD-2::GFP on its own (*left*) or in the presence of untagged MAD-2 (*right*) on monopolar spindles in 2-cell *C. elegans* embryos (see Fig. S1A for experimental details); all subsequent localization analysis of MAD-2::GFP was on monopolar spindles in the presence of untagged MAD-2. **(C,D)** MAD-2::GFP localization following introduction of structure-guided mutations in MAD-1's MAD-2 interaction motif (MIM) (*C*) and in MAD-2's dimerization interface (*D*). In this and all subsequent figures, *n* in graphs is number of embryos imaged and quantified. error bars are 95% CI, p-values (asterisks) are from Mann-Whitney tests. Scale bars, 2 μm.

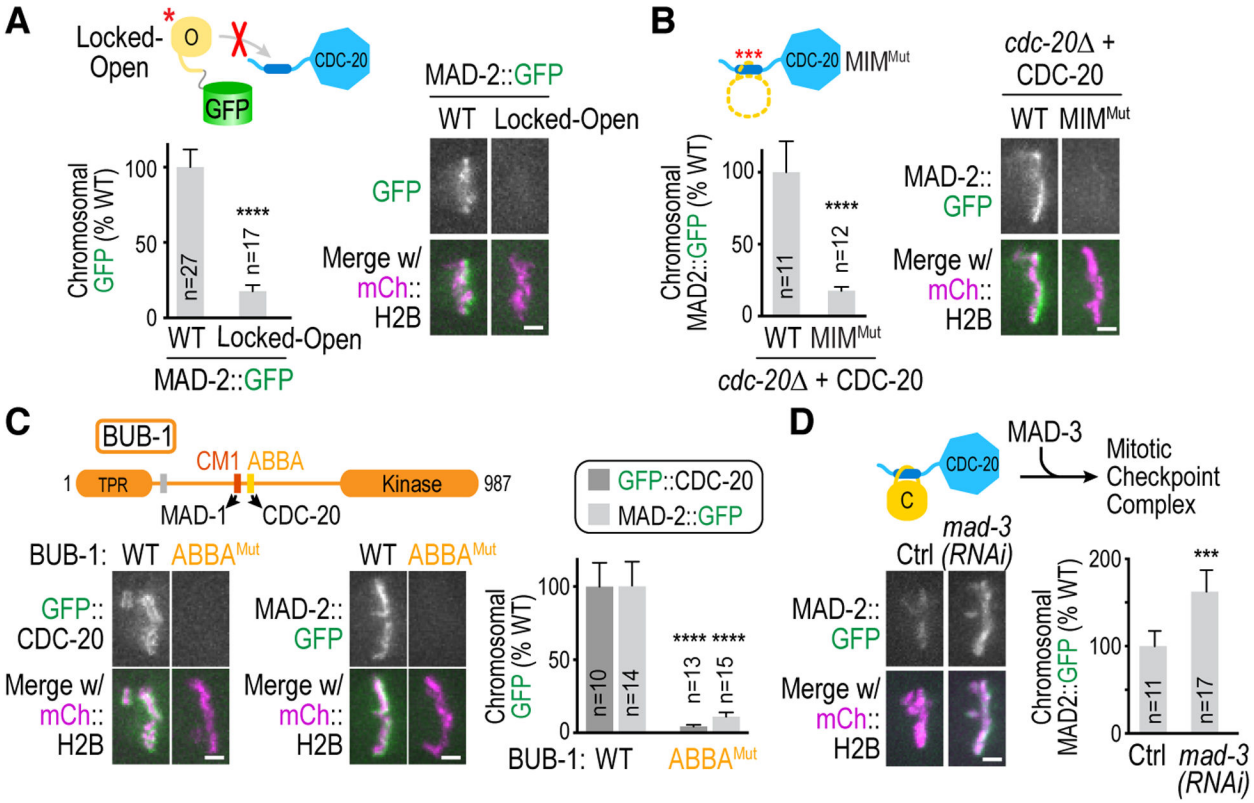

Figure 2: MAD-2::GFP reveals complex formation with CDC-20 at the kinetochore in living cells.

(A) (*left*) Schematic of a locked-open version of MAD-2, which exhibits normal dimerization but is unable to bind ligands, including CDC-20 (Fig. S2A). (*right*) Comparison of wildtype and locked-open MAD-2::GFP localization; data for wildtype is the same as in Fig. 1D. **(B)** MAD-2::GFP localization in the presence of wildtype or MAD-2 interaction motif-mutant (MIM^{Mut}) CDC-20. **(C)** (*top*) Schematic of BUB-1, whose ABBA motif binds to the CDC-20 WD40 domain. (*bottom*) GFP::CDC-20 and MAD-2::GFP localization in the presence of wildtype or ABBA-mutant BUB-1. **(D)** MAD-2::GFP localization following RNAi-mediated depletion of MAD-3. Scale bars, 2 μm.

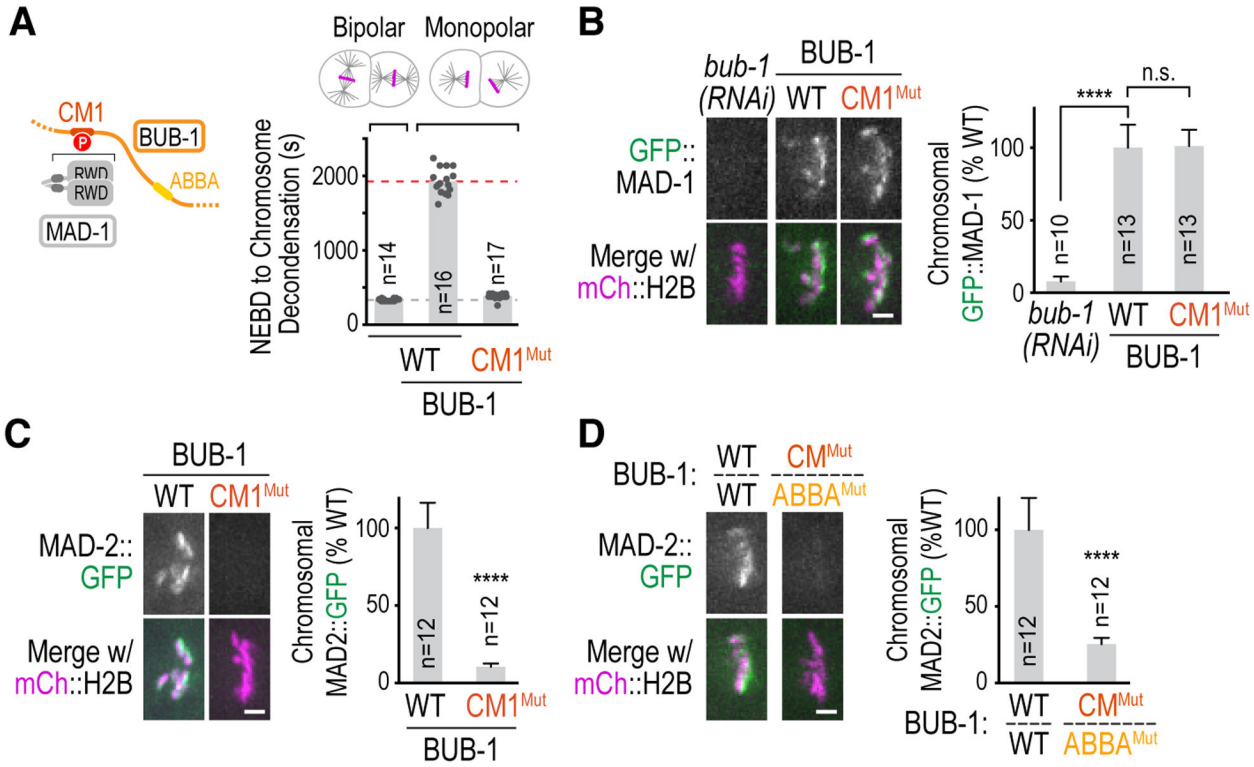

Figure 3: The conserved motif of Bub1 positions Cdc20 bound to Bub1 to engage with Mad2.

(A) (*left*) Schematic of Bub1 conserved motif (CM1), when phosphorylated, mediating an interaction with the C-terminus of Mad1. (*right*) Checkpoint signaling analysis, conducted by comparing mitotic duration in 2-cell embryos with bipolar (*grey dashed line*) versus monopolar (*red dashed line*) spindles (13). **(B)** & **(C)** Localization of GFP::MAD-1 (*B*) and MAD-2::GFP (*C*) in the indicated conditions. **(D)** MAD-2::GFP localization in a heterozygous *bub-1* mutant, with one allele expressing the CM1 mutant and the other allele expressing the ABBA mutant. *bub-1* mutations were engineered at the endogenous locus. Scale bars, 2 μm.

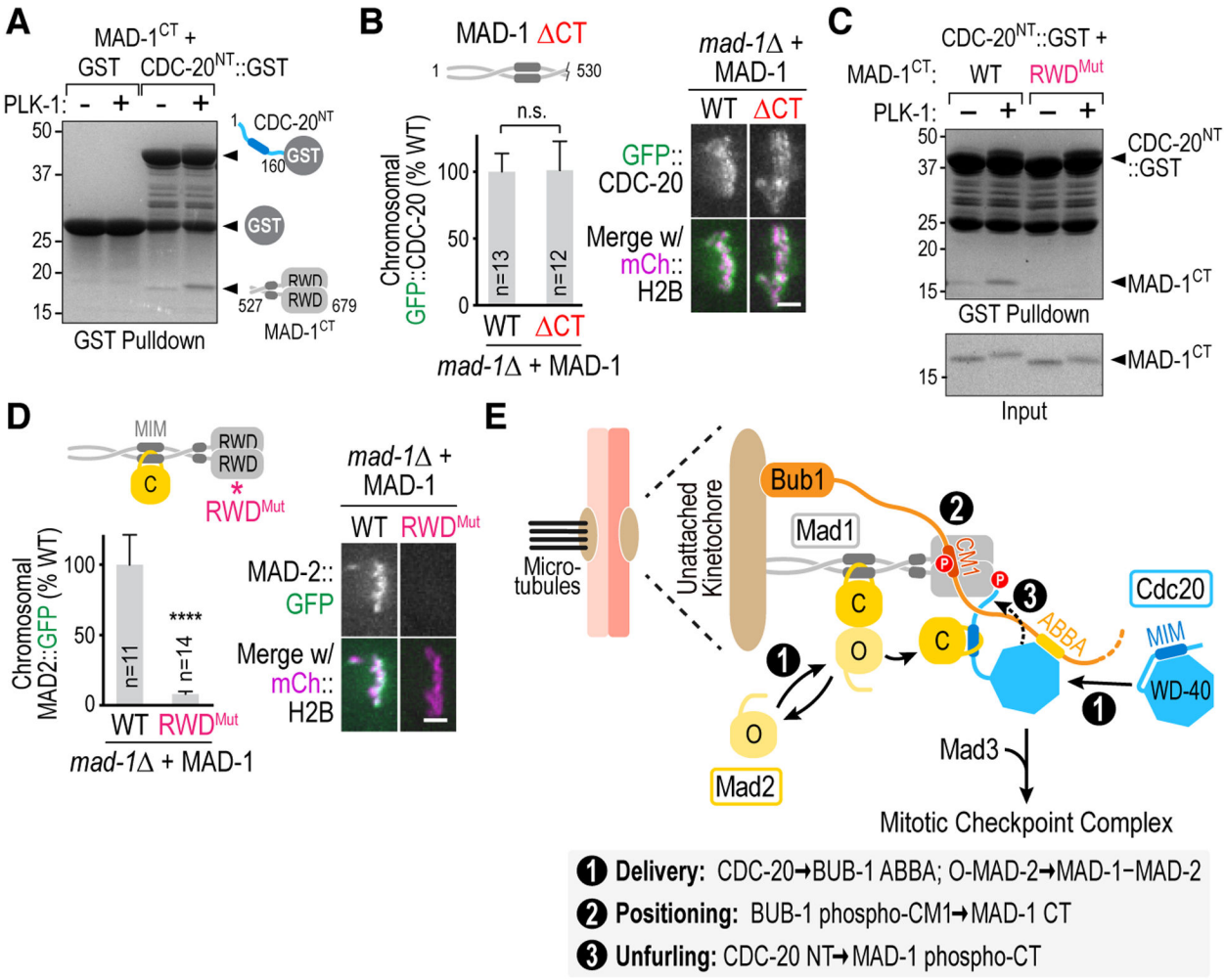

Figure 4: Phospho-regulated interaction of Mad1 and Cdc20 promotes Cdc20 association with Mad2.

(A) Coomassie-stained protein gel showing PLK-1 phosphorylation-promoted interaction of the MAD-1 C-terminus with the CDC-20 N-terminus. **(B)** CDC-20 localization in wild-type versus ΔCT MAD-1. **(C)** & **(D)** CDC-20 interaction (*C*), analyzed as in (*A*), and MAD-2::GFP localization (*D*) for wildtype versus RWD-mutant MAD-1. For details on residues mutated, see Fig. S7D. Scale bars, 2 μm. **(E)** Model of the tripartite mechanism that catalyzes localized Mad2–Cdc20 assembly at the kinetochore.