

[20] Purification and Assay of Mad2:
A Two-State Inhibitor of Anaphase-Promoting
Complex/Cyclosome

By XUELIAN LUO and HONGTAO YU

Abstract

To maintain the fidelity of chromosome inheritance, cells utilize a surveillance mechanism called the spindle checkpoint to sense improper attachment of sister chromatids to the mitotic spindle prior to chromosome segregation. The target of the spindle checkpoint is a ubiquitin ligase called the anaphase-promoting complex or cyclosome (APC/C). The spindle checkpoint protein Mad2 inhibits the activity of APC/C through direct binding to its activator Cdc20. Studies have shown that Mad2 has two distinct natively folded conformations and that the unusual two-state behavior of Mad2 plays a crucial role in checkpoint signaling. This article describes methods for the purification of the two Mad2 conformers and for the analysis of their activities in APC/C inhibition in *Xenopus* egg extracts.

Introduction

During a normal cell division cycle, the chromosomes are duplicated precisely once and then distributed evenly into two daughter cells (Nasmyth, 2002). The accuracy of this process plays a pivotal role in maintaining the genetic stability of the organism. Errors in chromosome segregation might promote aneuploidy in cells and lead to cancer formation (Jallepalli and Lengauer, 2001). Prior to anaphase, the sister chromatids are kept together by a protein complex termed cohesin (Nasmyth, 2002). After all the chromosomes are attached to the microtubules and are aligned at the cell equator, the anaphase-promoting complex or cyclosome, a multisubunit E3 ubiquitin ligase, tags the securin protein with polyubiquitin chains and results in its degradation by the proteasome (Harper *et al.*, 2002; Peters, 2002). The destruction of securin releases its inhibitory effect on separase, a cysteine protease of the CD clan family (Nasmyth, 2002). The activated separase then proteolyzes one of the cohesin subunits, Scc1, thus destroying the cohesion between the sister chromatids and allows the onset of anaphase (Nasmyth, 2002). APC/C is thus required indirectly for the initiation of sister chromatid separation.

To ensure the fidelity of chromosome segregation, cells employ a surveillance mechanism called the spindle checkpoint to monitor mistakes in the attachment of sister chromatids to the mitotic spindle before their separation (Bharadwaj and Yu, 2004; Musacchio and Hardwick, 2002). The molecular components of the spindle checkpoint include Bub1–3 and Mad1–3. APC/C is an important molecular target of the checkpoint (Yu, 2002). A single kinetochore that has not attached to the mitotic spindle in the cell can activate the spindle checkpoint (Rieder *et al.*, 1995). This suggests that an inhibitory signal is generated by this kinetochore to inhibit APC/C throughout the cell and block chromosome segregation (Yu, 2002). Although the nature of this diffusible “wait anaphase” signal is still unclear, the mitotic checkpoint complex (MCC) that contains BubR1 (Mad3), Bub3, Mad2, and Cdc20, and the subcomplexes of MCC, are likely candidates for this signal (Yu, 2002). In particular, it has been noted that the binding between Mad2 and Cdc20 is absolutely required for the proper function of the checkpoint and is enhanced greatly upon checkpoint activation. Mad2 also interacts with another checkpoint protein, Mad1, that recruits Mad2 to the kinetochore and is required for Mad2 binding to Cdc20 (Chen *et al.*, 1998, 1999). Moreover, p31^{comet}, a Mad2-binding protein identified through a yeast two-hybrid screen, has been implicated in the silencing of the checkpoint (Habu *et al.*, 2002; Xia *et al.*, 2004). Finally, phosphorylation of the C terminus of Mad2 also seems to regulate its function negatively (Wassmann *et al.*, 2003).

Recent structural studies have provided new insights into the interactions between Mad2 and its aforementioned binding partners (Musacchio and Hardwick, 2002). Mad2 recognizes similar short peptide motifs in Mad1 (its upstream regulator) and Cdc20 (its downstream target) (Luo *et al.*, 2002). Using nuclear magnetic resonance (NMR) techniques, we have determined the solution structures of free Mad2 and Mad2 in complex with MBP1, a peptide ligand identified using phage display that mimics the Mad2-binding sequences of both Mad1 and Cdc20 (Luo *et al.*, 2000, 2002). Based on our NMR structures, Mad2 undergoes a similarly dramatic conformational change upon binding to Mad1 or Cdc20. The Mad2-binding peptide of Mad1 or Cdc20 inserts as a central strand into the main β sheet of Mad2. The C-terminal region of Mad2 then moves across from one side of the β sheet to the other side to form two new strands. This unusual conformational change of Mad2 has also been observed in the crystal structure of Mad2 in complex with a 120 residue fragment of Mad1 (Sironi *et al.*, 2002).

Mad1 and Cdc20 bind to the same site on Mad2. Binding of Mad1 to Mad2 also induces the same conformational changes in Mad2 as does Cdc20. Thus, Cdc20 and Mad1 binding to Mad2 are mutually exclusive.

Consistently, overexpression of Mad1 in cells inhibits the function of Mad2 (Luo *et al.*, 2002). However, Mad1 is also required for the proper function of Mad2, as genetic deletion or RNAi-mediated depletion of Mad1 causes checkpoint defects in yeast or human cells, respectively. To explain this paradox of Mad1, we have proposed that Mad2 dissociated from Mad1 must transiently retain an activated conformation that is more suitable for subsequent Cdc20 binding (Yu, 2002). This hypothesis suggests that Mad2 might have a second conformation. Consistent with this notion, previous studies have shown that the bacterially expressed Mad2 protein exists as both monomeric and dimeric forms (Fang *et al.*, 1998). The dimeric Mad2 is more potent in inhibiting APC/C in *Xenopus* egg extracts than the monomeric form, suggesting that the dimeric Mad2 might have an activated conformation (Fang *et al.*, 1998).

To further study this activated conformation of Mad2 by NMR, we decided to use the Mad2 R133A mutant that was exclusively monomeric *in vitro* and yet retained the full biological activity of the wild-type Mad2 (Sironi *et al.*, 2001). To obtain the activated conformer of Mad2 R133A, we first expressed the protein in bacteria and showed that it indeed existed only as a monomer. However, when the Mad2 R133A protein was fractionated on an anion-exchange column, there were two well-separated peaks in the chromatograph (Fig. 1). We named the first low-salt peak N1-Mad2 R133A and the second high-salt peak N2-Mad2 R133A (Luo *et al.*, 2004). N1 and N2 stood for native fold 1 and 2, respectively. Based on two-dimensional NMR experiments, the structure of N1-Mad2 R133A was identical to that of the free Mad2 determined previously, whereas the structure of N2-Mad2 R133A resembled that of the conformation of Mad2 when bound to Mad1 or Cdc20 (Luo *et al.*, 2004). This was confirmed when we solved the structure of the N2-Mad2 R133A by NMR (Fig. 2) (Luo *et al.*, 2004). Indeed, N2-Mad2 R133A had a fold similar to that of Mad2 in complex with Cdc20, except with a vacant peptide-binding site. More surprisingly, we showed that N1-Mad2 R133A could spontaneously convert into N2-Mad2 R133A after an overnight incubation at room temperature (Luo *et al.*, 2004). Thus, the two conformers of Mad2 R133A exist in equilibrium with a large energetic barrier, and N2-Mad2 R133A is more stable than N1-Mad2 R133A *in vitro* (Luo *et al.*, 2004). The wild-type Mad2 also has N1 and N2 conformations and undergoes similar N1 to N2 conversion, except that the wild-type N2-Mad2 is a dimer (Luo *et al.*, 2004). Finally, we showed that the conversion from N1-Mad2 to N2-Mad2 is accelerated greatly by the Mad2-binding motif of Mad1, suggesting that Mad1 might facilitate the N1-N2 structural rearrangement of Mad2 (Luo *et al.*, 2004).

To determine the biological relevance of the two conformations of Mad2, we have tested the two Mad2 R133A conformers in an *in vitro*

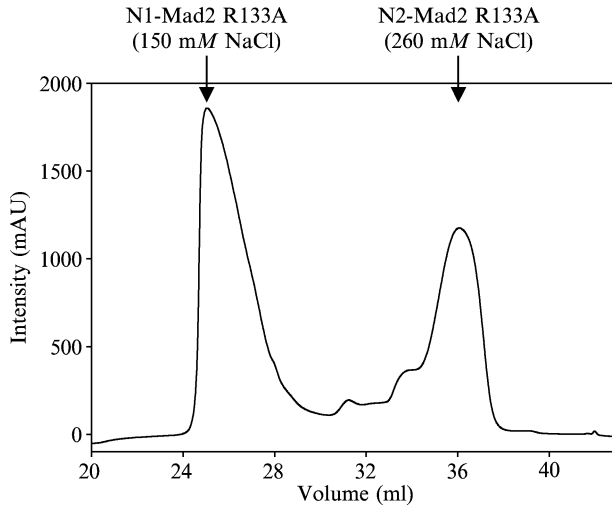


FIG. 1. The chromatograph of Mad2 R133A eluted from a Mono-Q column. The salt concentrations of the two peaks are labeled.

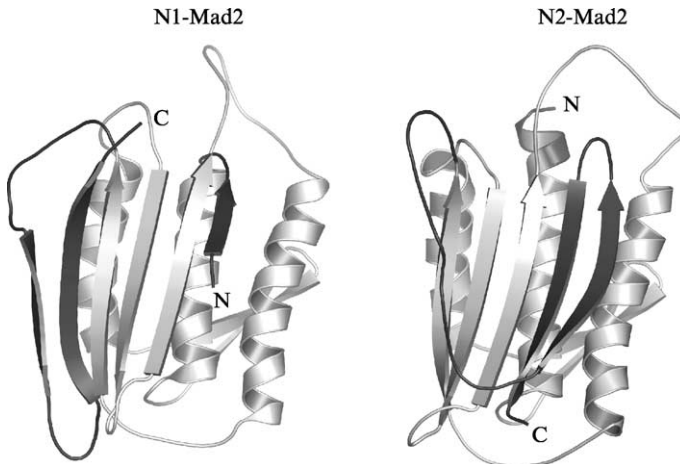


FIG. 2. Structures of N1-Mad2 (left) and N2-Mad2 (right). The C-terminal region of Mad2 that undergoes dramatic conformational changes between the N1 and the N2 conformers is shown as dark gray. N and C termini are labeled.

cyclin B1 degradation assay (Luo *et al.*, 2004). N2-Mad2 was more potent than N1-Mad2 in inhibiting APC/C and cyclin B degradation in *Xenopus* egg extracts (Luo *et al.*, 2004). The wild-type dimeric N2-Mad2 was also more active in blocking cyclin B1 degradation than the wild-type N1-Mad2 monomer (Fang *et al.*, 1998; Luo *et al.*, 2004). Interestingly, the wild-type N2-Mad2 dimer was only slightly more active than the N2-Mad2 R133A monomer, suggesting that dimerization itself is not required for efficient APC/C inhibition (Luo *et al.*, 2004). Thus, N2-Mad2 is very likely the activated form of Mad2. Our data are consistent with the notion that Mad1 facilitates the binding of Mad2 to Cdc20 by catalyzing formation of the N2-Mad2 conformer, which is more compatible for Cdc20 binding. This model explains the paradox that Mad1 is required for binding of Mad2 to Cdc20 *in vivo*, yet Mad1 acts as a competitive inhibitor of Cdc20 binding by Mad2. Thus, the ratio of Mad1/Mad2 seems crucial for proper spindle checkpoint signaling *in vivo*. Without Mad1, N2-Mad2 does not form efficiently, causing defects in the spindle checkpoint. However, over-expression of Mad1 results in the sequestration of Mad2, preventing its interaction with Cdc20.

In summary, our results suggest that the unusual two-state behavior of Mad2 is critical for spindle checkpoint signaling. The unique properties of Mad2 provide a clear example of how the conformational malleability of a protein might be used as a signaling mechanism for an important biological process. This chapter describes the detailed procedure for obtaining the two conformers of the recombinant human Mad2 protein and for assaying the APC/C inhibitory activities of these two conformers in crude *Xenopus* egg extracts.

Expression and Purification of N1- and N2-Mad2

The human Mad2 protein is overexpressed in *Escherichia coli* and is purified as an N-terminally His₆-tagged fusion protein. Due to the direct involvement of the C-terminal region of Mad2 in the conformational change, addition of any tags at the C terminus of Mad2 prevents the formation of N2-Mad2 and inhibits the biochemical function of Mad2. Using conventional recombinant DNA techniques, the coding region of the wild-type Mad2 protein was cloned into the *Bam*HI and *Hind*III sites of pQE30 (Qiagen, Valencia, CA) with a 5' primer encoding a tobacco etch virus (TEV) protease cleavage site. The pQE30-Mad2 R133A was made with the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The vectors were validated by DNA sequencing and then transformed into *E. coli* strain M15[pREP4] (Qiagen). The purification procedures for the wild-type Mad2 and the Mad2 R133A mutant are similar except that the

wild-type N2-Mad2 dimer elutes earlier from the gel-filtration column than the wild-type N1-Mad2 and Mad2 R133A monomers.

Materials

LB medium (GIBCO)
Carbenicillin (Sigma) stock solution (50 mg/ml)
Kanamycin (Sigma) stock solution (50 mg/ml)
Ampicillin (Sigma) stock solution (100 mg/ml)
Isopropyl- β -D-thiogalactoside (IPTG) (Sigma) (1 M)
AEBSF (Research Products International) (100 mM)
Protease inhibitor cocktail (P-2714, Sigma), stock concentration (100 \times): 10 mM AEBSF, 5 mM EDTA, 0.65 mM Bestatin, 7 μ M E-64, 5 μ M leupeptin, and 1.5 μ M aprotinin
Ni²⁺-NTA agarose (Qiagen)
Empty columns (Bio-Rad)
PD-10 column (Amersham)
Sonication buffer: 50 mM sodium phosphate (pH 7.8) + 300 mM NaCl
Wash buffer: 50 mM sodium phosphate (pH 6.0) + 300 mM NaCl + 10% glycerol
Elution buffer: 50 mM sodium phosphate (pH 6.0) + 300 mM NaCl + 10% glycerol + 150 mM imidazole
TEV cleavage buffer: 50 mM Tris (pH 8.0) + 100 mM NaCl + 1 mM dithiothreitol (DTT)
QA buffer: 20 mM Tris (pH 8.0) + 50 mM NaCl + 1 mM DTT
QB buffer: 20 mM Tris (pH 8.0) + 1 M NaCl + 1 mM DTT
Superdex-75 buffer: 50 mM sodium phosphate (pH 6.8) + 0.3 M KCl + 1 mM DTT

Procedures

1. Spread the M15[pREP4] cells that are freshly transformed with pQE30-Mad2 onto a LB agar plate containing 50 μ g/ml carbenicillin (Carb) and 50 μ g/ml kanamycin (KAN). Incubate the plate overnight at 37 $^{\circ}$.

2. Inoculate 100 ml of LB medium containing 100 μ g/ml ampicillin and 50 μ g/ml kanamycin in a 250-ml flask with a single colony from the LB/Carb/Kan agar plate. Grow the culture for 12 to 16 h at 37 $^{\circ}$ with vigorous shaking (250 rpm).

3. Inoculate 6 liter of LB medium containing 100 μ g/ml ampicillin and 50 μ g/ml kanamycin (1 liter each in six 2.8-liter flasks) by adding 10 ml of overnight culture to each flask. Incubate the six flasks of culture at 37 $^{\circ}$ with constant shaking (250 rpm) for about 2 h until reaching OD_{600nm} of 0.5–0.6.

Reduce the temperature to 16° and grow the bacterial culture for another 30 min.

4. Induce the expression of the fusion protein by adding IPTG to a final concentration of 0.2 mM. Grow the culture for additional 6–8 h at 16°.

5. Harvest the cells in 1-liter centrifuge bottles by centrifugation at 4000g at 4° for 20 min.

6. Discard supernatant and resuspend the cell pellets in 15 ml cold sonication buffer (pH 8.0) per liter of culture. Freeze sample in liquid N₂ and store at –80° until protein purification.

7. For protein purification, thaw cells in cold water for about 30 min. Add AEBSF, protease inhibitor cocktail, and β-mercaptomethanol to final concentrations of 0.2 mM, 1% (v/v), and 10 mM, respectively.

8. Lyse cells by passing the suspensions through an Emulsiflex-C5 homogenizer (Avestin, Canada) at a pressure of 75–150 MPa two times.

9. Pool the bacterial cell lysate into 50-ml centrifuge tubes and centrifuge at 30,000g at 4° for 45 min.

10. Collect and filter the supernatant through a 0.45-μm syringe filter. Add 12 ml of 50% slurry of Ni²⁺-NTA resin (preequilibrated with sonication buffer) to the cleared lysate. Rotate end over end at 4° for 90 min.

11. Load the mixture of the lysate and the Ni²⁺-NTA resin onto an empty column and collect the column flow through.

12. Wash the resins three times with 20 ml sonication buffer in each wash.

13. Wash the resins three times with 20 ml wash buffer each time.

14. Wash twice with 15 ml wash buffer containing 20 mM imidazole.

15. Elute the proteins from the Ni²⁺-NTA resins four times with 6 ml elution buffer each time. Collect the four elutions separately in 15-ml conical tubes.

16. Exchange the sample into TEV cleavage buffer by passing through PD–10 columns that have been preequilibrated with the TEV cleavage buffer.

17. Add TEV protease (1 OD₂₈₀ of TEV per 100 OD₂₈₀ of Mad2 fusion protein) and incubate the protein sample overnight at 4°.

18. Purify the cleaved Mad2 protein using an anion-exchange Mono-Q column with the Akta FPLC system (Amersham). The Mono-Q column is first equilibrated with 5 column volume (CV) of QA buffer. The Mad2 protein in QA buffer is loaded onto the column at a flow rate of 1 ml/min. Elution is performed with a 20 CV linear gradient of 0–40% (v/v) QB buffer.

19. Analyze the fractions by SDS–PAGE followed by Coomassie blue staining. Fractions containing N1-Mad2 and N2-Mad2 are pooled separately and concentrated to 5 ml.

20. Equilibrate a HiLoad 16/60 Superdex-75 column (Amersham) with two CV of Superdex-75 buffer. Load the two Mad2 conformers separately onto the column with a flow rate of 1 ml/min. Collect 3-ml fractions. Analyze the fractions by SDS-PAGE followed by Coomassie blue staining. Pool the fractions containing N1- and N2-Mad2 separately.

21. Concentrate the purified proteins to the desired concentration using Centriprep-10 (Millipore) at $<3000g$ at 4° . Freeze the samples in liquid nitrogen and store at -80° in small aliquots for further experiments.

Cyclin B Degradation Assay in *Xenopus* Egg Extracts

In this assay, we use *in vitro*-translated ^{35}S -labeled full-length human cyclin B1 as the APC/C substrate. To eliminate the possibility that some undetectable substances are copurified with N2-Mad2, but not with N1-Mad2, we have used N2-Mad2 converted from N1-Mad2 by incubating N1-Mad2 at 30° overnight in test tubes. However, for most applications, N2-Mad2 purified using the protocol described earlier can be used directly in the cyclin B degradation assay. All the experiments are done at room temperature. The amount of N1-Mad2 that has transformed into N2-Mad2 during the assays is negligible.

Materials

Mitotic $\Delta 90$ *Xenopus* egg extracts

Bovine ubiquitin (Sigma). Dissolve in H_2O at a final concentration of 10 mg/ml. Aliquot and store at -80° .

Energy mix (20 \times), 100 ml: 3.827 g phosphocreatine (150 mM final concentration) (Sigma), 1.102 g ATP (20 mM) (Sigma), 0.4 ml of 0.5 M EGTA (pH 7.7) (2 mM), and 2 ml of 1 M MgCl_2 (20 mM), adjust pH to 7.7, aliquot, and store at -80° .

XB buffer: 10 mM HEPES (pH 7.7), 100 mM KCl, 0.1 mM CaCl_2 , 1 mM MgCl_2 , and 50 mM sucrose

^{35}S -labeled human cyclin B1: *in vitro* translated in SP6-coupled rabbit reticulocyte lysate (Promega) in the presence of [^{35}S]methionine (Amersham) per manufacturer's protocols

SDS sample buffer

Procedures

1. Prepare mitotic $\Delta 90$ *Xenopus* egg extracts as described (Murray, 1991).
2. Thaw an aliquot of the N1-Mad2 protein in cold water. Divide the sample evenly into two Eppendorf tubes. Freeze one tube of N1-Mad2

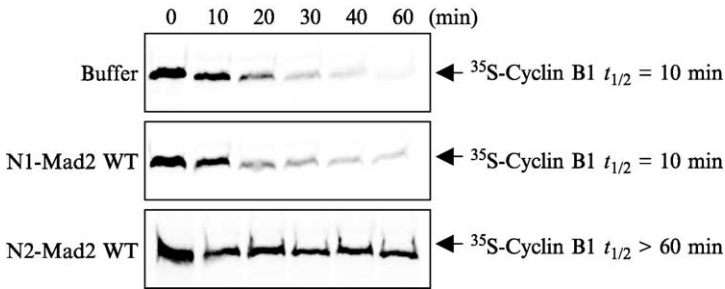


FIG. 3. Cyclin B degradation assay. The ^{35}S -labeled human cyclin B1 protein in reticulocyte lysate was added to mitotic $\Delta 90$ *Xenopus* egg extracts containing XB buffer, N1-Mad2 (0.5 mg/ml final concentration), or N2-Mad2 (0.5 mg/ml final concentration). Samples were taken at the indicated time points and separated by SDS-PAGE followed by Phosphorimager analysis.

with liquid nitrogen and store at -80° . This tube of N1-Mad2 will be used in the cyclin B degradation assay as the negative control. Incubate the other tube of N1-Mad2 in a 30° incubator for 18 h to allow it to convert into N2-Mad2.

3. Thaw the N1-Mad2 sample described in step 2 in cold water. Adjust the concentrations of N1-Mad2 and N2-Mad2 proteins to 5 mg/ml with XB buffer. Set up three 500- μl Eppendorf tubes. Add 37 μl of mitotic $\Delta 90$ *Xenopus* egg extracts to each tube. Add 5 μl XB to one tube, 5 μl N1-Mad2 protein to the second tube, and 5 μl N2-Mad2 protein to the third tube. Incubate at room temperature for 20 min.

4. Add 2.5 μl energy mix and 2.5 μl ubiquitin to each of the three reaction tubes. Add 3 μl ^{35}S -labeled cyclin B1 to each tube. Mix thoroughly. Incubate at room temperature. The total volume of each reaction is 50 μl .

5. Remove 3 μl of reaction mixture from each tube at 0, 10, 20, 30, 40, and 60 min. Mix immediately with 40 μl of SDS sample buffer.

6. After all time points have been completed, boil the samples for 5 min. Load 10 μl of each sample on a 12% SDS-PAGE gel. Dry the gel with a gel dryer. Expose the gel to a Phosphorimager plate and analyze the results with a phosphorimager (Fig. 3).

Acknowledgments

This work is supported by the National Institutes of Health (5 K01 CA100292 to X. L. and GM61542 to H. Y.), the Packard Foundation, the Burroughs Wellcome Fund, and the Robert A. Welch Foundation (I-1441).

References

- Bharadwaj, R., and Yu, H. (2004). The spindle checkpoint, aneuploidy, and cancer. *Oncogene* **23**, 2016–2027.
- Chen, R. H., Brady, D. M., Smith, D., Murray, A. W., and Hardwick, K. G. (1999). The spindle checkpoint of budding yeast depends on a tight complex between the Mad1 and Mad2 proteins. *Mol. Biol. Cell* **10**, 2607–2618.
- Chen, R. H., Shevchenko, A., Mann, M., and Murray, A. W. (1998). Spindle checkpoint protein Xmad1 recruits Xmad2 to unattached kinetochores. *J. Cell Biol.* **143**, 283–295.
- Fang, G., Yu, H., and Kirschner, M. W. (1998). The checkpoint protein MAD2 and the mitotic regulator CDC20 form a ternary complex with the anaphase-promoting complex to control anaphase initiation. *Genes Dev.* **12**, 1871–1883.
- Habu, T., Kim, S. H., Weinstein, J., and Matsumoto, T. (2002). Identification of a MAD2-binding protein, CMT2, and its role in mitosis. *EMBO J.* **21**, 6419–6428.
- Harper, J. W., Burton, J. L., and Solomon, M. J. (2002). The anaphase-promoting complex: It's not just for mitosis any more. *Genes Dev.* **16**, 2179–2206.
- Jallepalli, P. V., and Lengauer, C. (2001). Chromosome segregation and cancer: Cutting through the mystery. *Nature Rev. Cancer* **1**, 109–117.
- Luo, X., Fang, G., Coldiron, M., Lin, Y., Yu, H., Kirschner, M. W., and Wagner, G. (2000). Structure of the Mad2 spindle assembly checkpoint protein and its interaction with Cdc20. *Nature Struct. Biol.* **7**, 224–229.
- Luo, X., Tang, Z., Rizo, J., and Yu, H. (2002). The Mad2 spindle checkpoint protein undergoes similar major conformational changes upon binding to either Mad1 or Cdc20. *Mol. Cell* **9**, 59–71.
- Luo, X., Tang, Z., Xia, G., Wassmann, K., Matsumoto, T., Rizo, J., and Yu, H. (2004). The Mad2 spindle checkpoint protein has two distinct natively folded states. *Nature. Struct. Mol. Biol.* **11**, 338–345.
- Murray, A. W. (1991). Cell cycle extracts. *Methods Cell Biol.* **36**, 581–605.
- Musacchio, A., and Hardwick, K. G. (2002). The spindle checkpoint: Structural insights into dynamic signalling. *Nature Rev. Mol. Cell Biol.* **3**, 731–741.
- Nasmyth, K. (2002). Segregating sister genomes: The molecular biology of chromosome separation. *Science* **297**, 559–565.
- Peters, J. M. (2002). The anaphase-promoting complex: Proteolysis in mitosis and beyond. *Mol. Cell* **9**, 931–943.
- Rieder, C. L., Cole, R. W., Khodjakov, A., and Sluder, G. (1995). The checkpoint delaying anaphase in response to chromosome monoorientation is mediated by an inhibitory signal produced by unattached kinetochores. *J. Cell Biol.* **130**, 941–948.
- Sironi, L., Mapelli, M., Knapp, S., Antoni, A. D., Jeang, K. T., and Musacchio, A. (2002). Crystal structure of the tetrameric Mad1-Mad2 core complex: Implications of a 'safety belt' binding mechanism for the spindle checkpoint. *EMBO J.* **21**, 2496–2506.
- Sironi, L., Melixetian, M., Faretta, M., Prosperini, E., Helin, K., and Musacchio, A. (2001). Mad2 binding to Mad1 and Cdc20, rather than oligomerization, is required for the spindle checkpoint. *EMBO J.* **20**, 6371–6382.
- Wassmann, K., Liberal, V., and Benezra, R. (2003). Mad2 phosphorylation regulates its association with Mad1 and the APC/C. *EMBO J.* **22**, 797–806.
- Xia, G., Luo, X., Habu, T., Rizo, J., Matsumoto, T., and Yu, H. (2004). Conformation-specific binding of p31(comet) antagonizes the function of Mad2 in the spindle checkpoint. *EMBO J.* **23**, 3133–3143.
- Yu, H. (2002). Regulation of APC-Cdc20 by the spindle checkpoint. *Curr. Opin. Cell Biol.* **14**, 706–714.