Supporting Information

Kim et al. 10.1073/pnas.1009000107

SI Materials and Methods

Flow Cytometry (FACS). For FACS analysis, cells were collected by trypsinization, washed once with PBS, and fixed with cold 70% ethanol. After overnight fixation at −20 °C, cells were washed with PBS and permeabilized with a 5-min incubation in PBS plus 0.25% (v/v) Triton X-100. Cells were then incubated with α-MPM2 (Millipore) followed by an incubation with fluorescent secondary antibodies (Invitrogen) diluted in PBS plus 1% BSA. After washing with PBS, the cells were stained with propidium iodide (Sigma) at a final concentration of 20 μg/ml; and simultaneously treated with 200 μg/ml RNAase A (Qiagen). The flow cytometry reading was performed with FacsScan (Becton Dickinson). The results were analyzed with the FlowJo software (Tree Star).

Metabolic ^{32}P Labeling. About 6×10^5 HeLa Tet-On cells were transfected with pCS2-Myc Mad2-wt or Mad2^{S195A} for 22 hrs and treated with nocodazole for another 14 hrs. The cells were then released into the labeling medium (phosphate-depleted DMEM plus 5% dialyzed FBS) for 2 hrs followed by another 2-hr incubation in the labeling medium plus ^{32}P-orthophosphate (0.3–0.5 mCi/60-mm plate; PerkinElmer). The cells were then lysed with the RIPA buffer (1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 10 mM sodium phosphate, pH 7.2, 2 mM EDTA, 50 mM sodium fluoride, 0.2 mM sodium vanadate, and 1X protease inhibitor cocktail). The Myc-Mad2 proteins were immunoprecipitated from the cleared cell lysate with α-Myc antibody (Roche), separated on SDS/PAGE, and transferred to a nitrocellulose membrane. The incorporation of ^{32}P was measured with a phosphorimager (FLA-5100; Fujifilm). The same membrane was blotted with α-Mad2 to compare the total amounts of precipitated proteins.

Intein-mediated Protein Ligation. The cDNA encoding the Mad2N fragment (residues 1–186) was amplified by PCR and cloned into pTXB1 (New England Biolabs) between the NdeI and SapI sites. Expression of pTXB1-Mad2N in BL21(DE3) produced Mad2N-intein-CBD fusion protein, which was bound to chitin beads. Chitin beads were washed with the ligation buffer (50 mM HEPES, pH 7.5, 250 mM NaCl). The ligation peptide containing residues 187–205 of Mad2, except that T187 was replaced with a cysteine and S195 with a phospho-serine, was chemically synthesized. The Mad2N-intein-CBD fusion protein on chitin beads was incubated with 1 mM ligation peptide and 60 mM 2-mercaptoethanesulfonic acid (MESNA) in the ligation buffer at room temperature overnight. The ligation product was eluted from the beads and further purified using anion exchange and size exclusion chromatography. Based on mass spectrometry, the final ligation product contained residues 2-205 of Mad2 with the expected T187C mutation and phospho-S195. The first methionine of Mad2N was presumably removed by bacterial aminopeptidases.

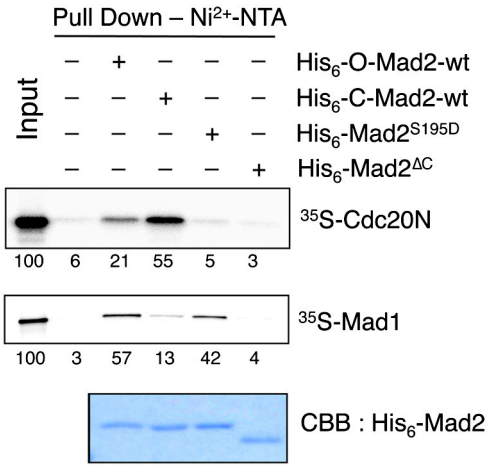

Fig. S1. Blank Ni^{2+}-NTA beads or beads bound to the indicated His$_6$-tagged Mad2 proteins were incubated with ^{35}S-labeled Mad1 or Cdc20N. After washing, the proteins retained on the beads were analyzed by SDS/PAGE followed by autoradiography. About 25% of the ^{35}S-labeled proteins used in the binding assays were loaded as input. Mad2 proteins bound to beads were also stained with Coomassie. The relative amounts of Mad1 or Cdc20N bound to beads were quantified.

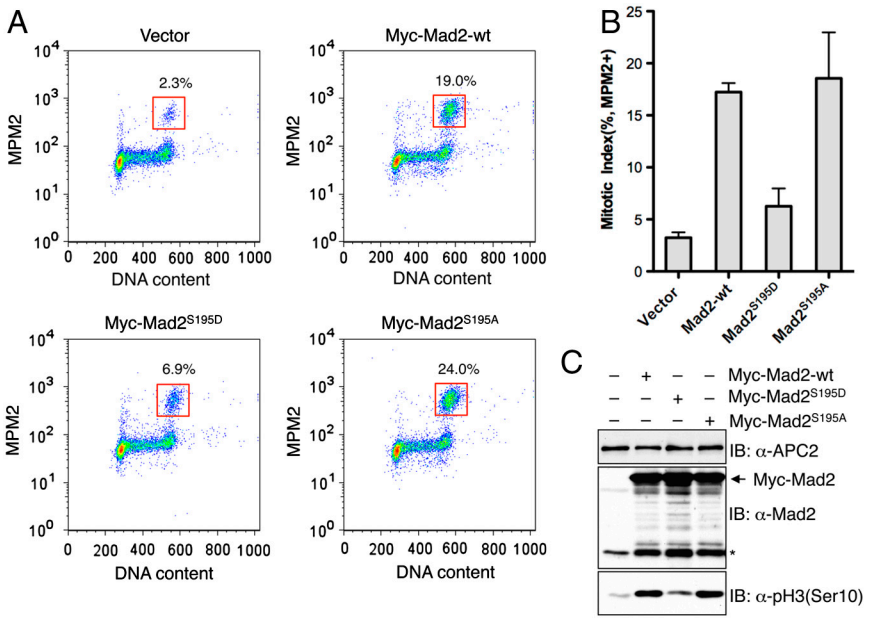

Fig. S2. Mad2^{S195D} is ineffective in eliciting mitotic arrest of human cells. (*A*) The FACS analysis of HeLa Tet-On cells transfected with the indicated plasmids. The populations of mitotic cells (with 4N DNA content and MPM2+) are boxed with the mitotic indices indicated. (*B*) Quantification of mitotic indices of cells described in *A*. The means and standard deviations of results from seven experiments are shown. (*C*) Lysates of cells described in *A* were blotted with the indicated antibodies. APC2 is used as the loading control. The asterisk indicates the position of the endogenous Mad2. Cells transfected with Myc-Mad2 proteins contained more Mad2 that comigrated with endogenous Mad2. The origins of these Mad2 species were unknown but might be due to internal translation start of Myc-Mad2 transgenes or the proteolysis of Myc-Mad2 proteins or both.

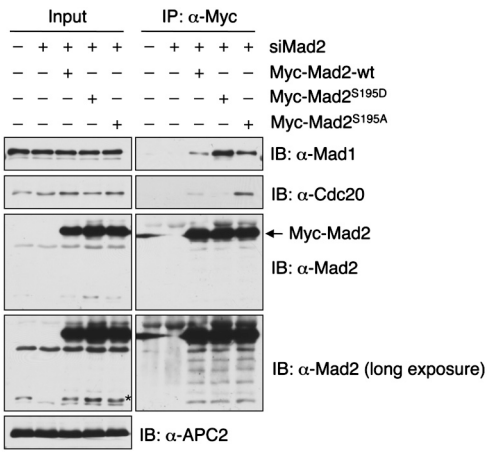

Fig. S3. Mad1 binding of Mad2^{S195D} does not require the endogenous Mad2. HeLa Tet-On cells were transfected with the indicated siRNA and Mad2 plasmids that contained silent mutations within the siRNA target sequence and treated with nocodazole. Lysates of these cells and the α-Myc immunoprecipitates were blotted with the indicated antibodies. The asterisk indicates the position of the endogenous Mad2 and the exogenous Mad2 that has lost the Myc tag.