ml)] on ice for 10 min followed by centrifugation at 10,000*g* for 5 min at 4°C. Proteins (20 μg) from the supernatants were loaded in each lane and an immunoblot was prepared with ECL (Amersham).
25. To make recombinant XMAD2, we tagged the full-length XMAD2 coding sequence with six histidines at the NH_2-terminus and expressed it in *Escherichia coli* with Qiaexpress vector pQE10 (Qiagen). The protein was purified according to the manufacturer's instructions. The purified proteins were dialyzed against a buffer containing 50 mM Hepes (pH 7.4), 50 mM potassium chloride, and 50% glycerol.
26. Freshly prepared CSF-arrested extracts (20 μl) were first incubated for 1 hour on ice with 1 μl (0.7 μg) of various antibodies. To block anti-XMAD2, we incubated the antibodies with 6H-XMAD2 (antibody:protein = 3:2 by weight or 1:2 in molar ratio) on ice for 1 hour. The extracts were then incubated with a low or high concentration (3000 or 10,000 nuclei per microliter, respectively) of demembranated sperm nuclei prepared as described (*22*) for 10 min at room temperature and for another 10 min at room temperature with nocodazole (10 μg/ml). The nocodazole stock was made in dimethyl sulfoxide at 10 mg/ml and was diluted 1:50 (200 μg/ml) in CSF-arrested extract before it was added to the reaction mixture. CSF-arrested extracts were incubated with a high concentration of sperm nuclei and nocodazole for 20 min and then with control antibodies or anti-XMAD2 at room temperature for 30 min. Calcium chloride (0.4 to 0.6 mM) was added to induce exit from mitosis and 1-μl samples were taken every 15 min and left on dry ice until all of the samples were ready for histone H1 kinase activity measurement as described (*22*).
27. XTC cells were fixed in 3% paraformaldehyde, and immunofluorescent staining was done as described (*21*), except that all buffers were made in 70% phosphate-buffered saline (PBS). For detergent extraction, cells were first extracted with 70% PBS with 0.5% Triton X-100 (PBST) for 5 min followed by fixation with paraformaldehyde. The images were viewed with a Nikon Microphot-FXA microscope and photographed with Kodak Tri-X pan 400 film.
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29. Cells were first simultaneously extracted and fixed with 0.5% Triton X-100, 2% formaldehyde in PHEM [60 mM Pipes, 25 mM Hepes (pH 7), 10 mM EGTA, 2 mM magnesium chloride] for 5 min and then fixed further in 4% formaldehyde in PHEM for 15 min. Immunofluorescent staining was then performed as described (*19*), except that the buffer PBS plus 0.05% Tween 20 was used. Z-series optical sections (0.5 μm thick) were captured with a multimode digital fluorescence microscope system. A Nikon Microphot-FXA microscope equipped with a ×60, numerical aperture 1.4 objective was used.
30. We thank A. Desai and T. Mitchison for the CREST serum and for their expertise and encouragement; J. Minshull for the *Xenopus* ovary cDNA library; R. Benezra, Y. Li, and T. Gustafson for sharing unpublished results; and T. Mitchison, D. Morgan, A. Desai, and members of A.W.M.'s laboratory for their helpful comments on the manuscript. Supported by grants from NIH, the Packard Foundation, the Markey Foundation, and the March of Dimes to A.W.M. R.-H.C. is a Helen Hay Whitney postdoctoral fellow.

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Identification of a Human Mitotic Checkpoint Gene: *hsMAD2*

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In *Saccharomyces cerevisiae*, *MAD2* is required for mitotic arrest if the spindle assembly is perturbed. The human homolog of *MAD2* was isolated and shown to be a necessary component of the mitotic checkpoint in HeLa cells by antibody electroporation experiments. Human, or *Homo sapiens*, MAD2 (hsMAD2) was localized at the kinetochore after chromosome condensation but was no longer observed at the kinetochore in metaphase, suggesting that MAD2 might monitor the completeness of the spindle-kinetochore attachment. Finally, T47D, a human breast tumor cell line that is sensitive to taxol and nocodazole, had reduced MAD2 expression and failed to arrest in mitosis after nocodazole treatment. Thus, defects in the mitotic checkpoint may contribute to the sensitivity of certain tumors to mitotic spindle inhibitors.

During mitosis, the onset of anaphase is demarcated by the separation of sister chromatids and the destruction of cyclin B, which are irreversible events that commit a cell to complete the division cycle (*1*). Mitotic checkpoint control mechanisms (*2*) test the cell's preparedness to undergo division and block cell cycle progression before the irreversible events associated with anaphase if the mitotic spindle apparatus is not appropriately assembled (*3*, *4*) or if the kinetochore is not properly attached to the spindle (*5*). In budding yeast, six nonessential genes have been identified that are required for the execution of the mitotic checkpoint: *MAD1*, *MAD2*, and *MAD3* (*3*), and *BUB1*, *BUB2*, and *BUB3* (*4*). These genes were identified in screens for mutants that are hypersensitive to drugs that inhibit mitotic spindle assembly by depolymerizing microtubules. A similar mitotic checkpoint pathway also exists in higher eukaryotes (*5*), but its molecular components have not yet been identified.

We isolated a human cDNA clone (*6*) in a screen for high copy number suppressors of thiabendazole (a mitotic spindle assembly inhibitor) sensitivity in yeast cells lacking CBF1, a component of the kinetochore (*7*). Sequence determination of the cDNA revealed an open reading frame (ORF) of 205 amino acids (GenBank accession number U65410) that is similar to that of the product of the mitotic checkpoint gene *MAD2* from the budding yeast, *S. cerevisiae*. The two proteins are 40% identical and 60% similar over the entire ORF (Fig. 1A). We therefore refer to the human MAD2 homolog as hsMAD2. The hsMAD2 protein is also similar to a MAD2 homolog from *Xenopus laevis*, termed XMAD2 (*8*) (Fig. 1A). The protein encoded by hsMAD2 cDNA has a predicted molecular size of 23.5 kD with two putative amphipathic α helices at residues 64 to 74 and 124 to 134. The fact that both *hsMAD2* and yeast *MAD2*, termed *scMAD2*, can partially suppress the thiabendazole sensitivity of *cbf1* null yeast cells (*9*) suggests that without *CBF1*, the mitotic checkpoint is not fully activated.

To further characterize hsMAD2, we generated polyclonal antibodies and affinity-purified them (*10*). Immunoglobulin G (IgG) fractions from preimmune serum and serum raised against hsMAD2 that was first passed over an hsMAD2 affinity column (referred to as anti-hsMAD2Δ) were also isolated (*10*). Protein immunoblot analysis (*11*) revealed that the affinity-purified anti-hsMAD2 specifically recognized a single protein of approximately 24 kD in HeLa cell extracts that was not detected with either preimmune IgG or anti-hsMAD2Δ (Fig. 1B). Extracts from HeLa cells that had been transiently transfected with an expression vector that encodes hsMAD2 showed increased intensity of the 24-kD band, indicating that this protein is probably encoded by the hsMAD2 cDNA (Fig. 1B). Thus, the affinity-purified antibody to hsMAD2 was highly specific for the protein expressed in human cells.

To determine if *hsMAD2* functions as a mitotic checkpoint gene, we introduced affinity-purified anti-hsMAD2 into HeLa cells by electroporation and determined the status of the mitotic checkpoint (*12*). When cells were electroporated with either the preimmune IgG, the anti-hsMAD2Δ, or buffer alone, many of them became rounded after nocodazole treatment, which is indicative of cells arrested in mitosis (Fig. 2). In contrast, cells electroporated with anti-hsMAD2 showed fewer rounded cells after nocodazole treatment (~80 to 90% of the cells that survived the electroporation took up IgG, as assayed by immunofluorescence). We measured the percentage of IgG^+ cells in mitosis (the mitotic index, MI) after nocodazole treatment of the electroporated cells. The average MI of the IgG^+ cells electroporated with either the preimmune IgG or the anti-hsMAD2Δ was about 30% (471 of 1588

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cells), whereas the MI of IgG^+ cells electroporated with anti-hsMAD2 was 1.8% (18 of 1016 cells) (Table 1). This latter result is unlikely to indicate arrest of the cells before the onset of mitosis because cells electroporated with anti-hsMAD2 continue to cycle for 30 hours at the same rate as the cells electroporated with the preimmune IgG (9). Thus, hsMAD2 is required in HeLa cells for the execution of the mitotic checkpoint in response to nocodazole treatment. Because XMAD2 is also an essential component of the mitotic checkpoint in *X. laevis* (8), we conclude that the mitotic checkpoint function of MAD2 is highly conserved during evolution and probably has a critical role in ensuring accurate chromosome segregation.

Previous studies (*5*, *13*) have pointed to a close link between the kinetochore and the mitotic checkpoint pathway. Therefore, we determined the subcellular localization of hsMAD2 in HeLa cells (*14*). During interphase, hsMAD2 was distributed throughout the cell, with prominent perinuclear localization (Fig. 3A). In mitotic cells, the pattern of hsMAD2 staining varied with the stage of mitosis. In prometaphase, hsMAD2 colocalized with the

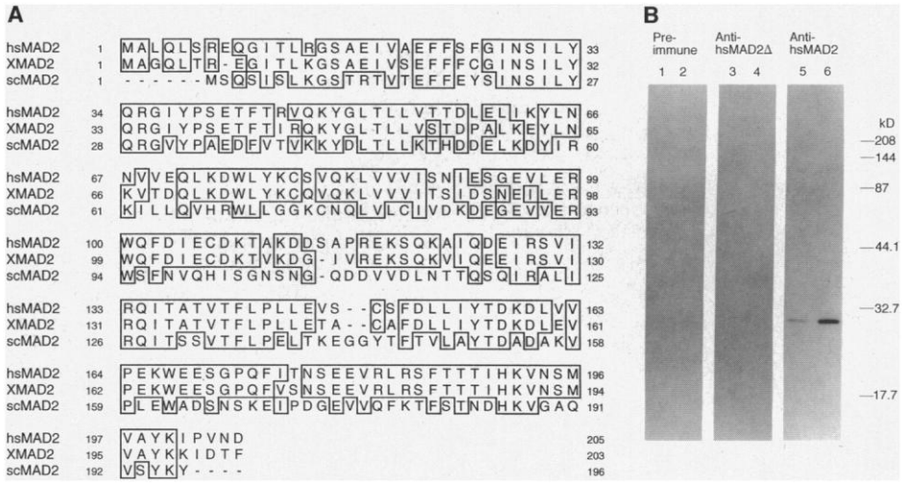

Fig. 1. Characterization of human MAD2. (**A**) Alignment of the predicted hsMAD2 protein sequence with those of *X. laevis* MAD2 (XMAD2) and *S. cerevisiae* MAD2 (scMAD2). Amino acids identical in at least two of the three MAD2 proteins are boxed. Dashes indicate gaps. Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr. (**B**) Human *MAD2* encodes a 24-kD protein. Protein extracts from HeLa cells (lanes 1, 3, and 5) or HeLa cells transiently transfected with pCMV5-hsMAD2 for 48 hours (lanes 2, 4, and 6) were subjected to protein immunoblot analysis with the antibodies indicated on top of each lane. The positions of prestained molecular size markers (Bio-Rad) are shown.

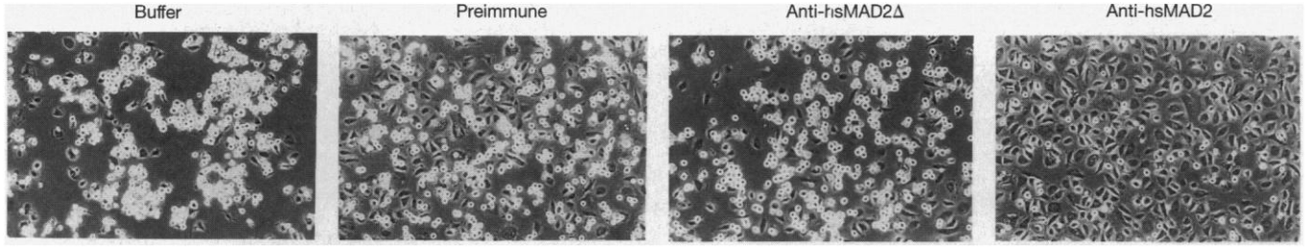

Fig. 2. Function of *hsMAD2* as a mitotic checkpoint gene. HeLa cells electroporated with anti-hsMAD2 failed to arrest in mitosis in the presence of nocodazole. HeLa cells were electroporated with buffer or antibodies as indicated (*12*). Electroporated cells were allowed to attach to the plates for 6 hours and then treated with 200 nM nocodazole for 18 hours before being photographed. Summary of the antibody electroporation experiments is found in Table 1.

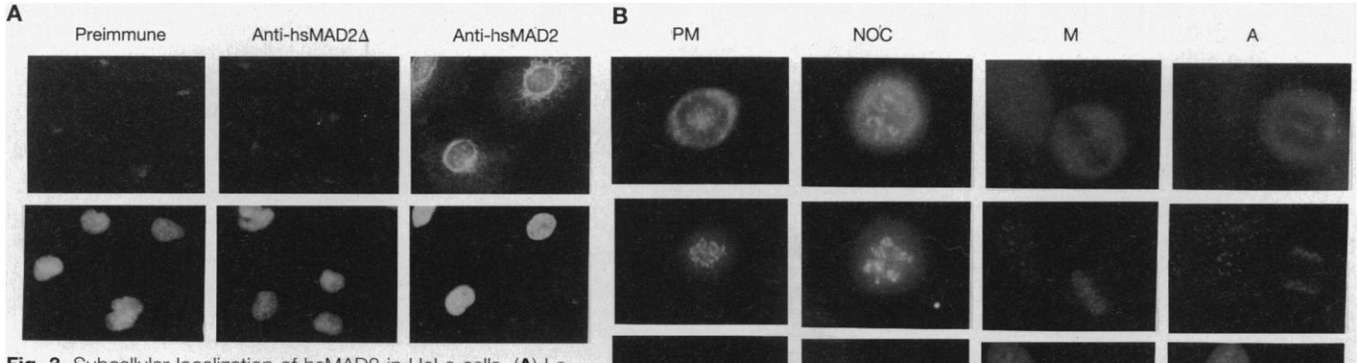

Fig. 3. Subcellular localization of hsMAD2 in HeLa cells. (**A**) Localization of hsMAD2 during interphase. HeLa cells were stained with the antibodies as indicated (top row). DNA was visualized with DAPI (bottom row). Cells were observed with a ×40 oil immersion objective. (**B**) Localization of hsMAD2 during mitosis. HeLa cells were triple-stained with affinity-purified anti-hsMAD2 (top row), human antibody to centromeres (middle row), and DAPI (bottom row). Cells in prometaphase (PM), arrested in prometaphase by nocodazole treatment (NOC), in metaphase (M), and in anaphase (A) are shown. Cells were observed with a ×100 oil immersion objective.

kinetochores (Fig. 3B). At metaphase and anaphase, however, hsMAD2 staining was absent from the chromosomes (Fig. 3B), which could be due to either the loss of hsMAD2 antigen from the kinetochore or epitope masking. The kinetochore localization of hsMAD2 in prometaphase, when most, but not all of the kinetochores are attached to the spindle, suggests that hsMAD2 may monitor the completeness of the spindle-kinetochore attachment and activate the mitotic checkpoint when this process is incomplete. Indeed, we observed persistent kinetochore localization of hsMAD2 in HeLa cells arrested in mitosis by nocodazole treatment in which spindle-kinetochore interaction is inhibited (*15*) (Fig. 3B).

Table 1. Summary of the antibody electroporation experiments (Fig. 2). Electroporated cells were treated with 200 nM nocodazole for 18 hours.

Experiment	Antibody	IgG$^+$ cells	IgG$^+$ mitotic cells
1*	Preimmune	254	52
	Anti-hsMAD2	206	3
2	Preimmune	270	77
	Anti-hsMAD2	295	6
3	Preimmune	261	83
	Anti-hsMAD2Δ	233	74
	Anti-hsMAD2	217	4
4	Preimmune	275	94
	Anti-hsMAD2Δ	295	91
	Anti-hsMAD2	298	5

*Cells were treated with 100 nM nocodazole for 12 hours.

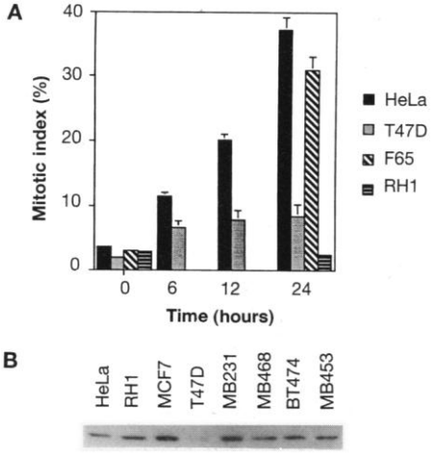

Fig. 4. Failure of T47D cells to arrest in mitosis in response to nocodazole treatment and expression of low amounts of hsMAD2. (**A**) T47D and RH1 cells failed to undergo mitotic arrest after nocodazole treatment. HeLa, F65, T47D, and RH1 cells were treated with 100 nM nocodazole and collected at the indicated times. F65 and RH1 were not sampled at 6 and 12 hours. Cells were transferred to slides and scored for their mitotic indices (*12*). (**B**) Reduced expression of hsMAD2 in T47D cells. Equal amounts of protein extracts from the indicated cell lines were subjected to protein immunoblot analysis with anti-hsMAD2.

Because yeast cells defective in mitotic checkpoint genes are sensitive to mitotic spindle inhibitors, we sought to determine if this was also true in mammalian cell lines. Two human tumor cell lines, T47D (breast cancer) and RH1 (rhabdomyosarcoma), are sensitive to taxol and nocodazole (9). Both cell lines failed to undergo mitotic arrest (Fig. 4A) and continued to divide 24 hours after nocodazole treatment (9), which suggests that they are defective in the mitotic checkpoint. No such failure to arrest was observed in HeLa cells or human primary diploid fibroblast cells (F65) (Fig. 4A), which are resistant to nocodazole (9). Thus, defects in the mitotic checkpoint may contribute to the sensitivity of certain tumors to mitotic spindle inhibitors. We also found, by protein immunoblot analysis, that T47D cells had less than one-third the amount of hsMAD2 that was present in nocodazole- and taxol-resistant cell lines (Fig. 4B). RH1 cells showed no such decrease. Therefore, the decrease in hsMAD2 in T47D cells may contribute to the failure of these cells to execute the mitotic checkpoint.

MAD proteins in budding yeast are required for accurate chromosome segregation under normal growth conditions (*3*). Therefore, loss of hsMAD2 function might also lead to aberrant chromosome segregation in mammalian cells, an event that is associated with tumor formation in a number of cell types (*16*). This hypothesis can now be tested by the generation and analysis of *MAD2* null mice.

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6. YNN415 (the *cbf1* null strain) was transformed with a human cDNA library, by using a lithium acetate method (*17*). Approximately 4×10^5 transformants were plated on YPD (yeast extract–peptone–dextrose) plates containing thiabendazole (100 μg/ml) (Sigma). After a 6-day incubation at 30°C, 19 clones were isolated and retested for thiabendazole resistance. The thiabendazole resistance of one clone was dependent on the plasmid bearing the human cDNA. Plasmid DNA was isolated from this clone and sequenced with Sequenase (U.S. Biochemical).
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10. Full-length hsMAD2 ORF was subcloned into pET-28a(+). The hsMAD2 tagged with six histidine residues (His_6) was overexpressed in BL21 and purified on a Ni^{2+}–nitrilotriacetic acid column according to manufacturer instructions (Qiagen). Polyclonal antibodies were prepared by injection of the purified fusion protein into two female New Zealand White rabbits (HRP, Pennsylvania). Purified His_6-tagged hsMAD2 was coupled to CNBr-activated Sepharose 4B (Pharmacia). The polyclonal antiserum to hsMAD2 was affinity-purified as described (*18*). The flow-through of the hsMAD2 affinity column was saved and passed over a protein A–Sepharose column (Pharmacia), and the IgG fraction was eluted to generate anti-hsMAD2Δ (*18*). IgG from the corresponding preimmune serum was also isolated. Purified IgGs were extensively dialyzed against phosphate-buffered saline (PBS) and concentrated to 2 mg/ml (anti-hsMAD2 and anti-hsMAD2Δ) or 1.5 mg/ml (preimmune IgG) in Centricon-30 units (Amicon).
11. Protein extracts were prepared by lysing cells in Nonidet P-40 (NP-40) lysis buffer [50 mM tris (pH 7.5), 150 mM NaCl, 1% NP-40, 10% glycerol, 2 mM EDTA, 50 mM NaF, 0.25 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, and a final concentration of 5 μg/ml for each of the following: aprotinin, antipain, pepstatin, and leupeptin]. Protein extracts were resolved by SDS–polyacrylamide gel electrophoresis (12% gel) and transferred to nitrocellulose membranes (Bio-Rad) (*18*). Immunoblotting was done with the enhanced chemiluminescence protocol (DuPont NEN). The affinity-purified antibodies were used at 0.8 μg/ml.
12. HeLa cells (1×10^6) were incubated in 100 μl of PBS containing 25 μg of affinity-purified antibodies for 10 min at room temperature in 0.4 cm Gene Pulser cuvettes. The electric pulse was delivered from a Gene Pulser (Bio-Rad) set at 300 V, infinite resistance, and 250 μF. Cells were then transferred into warm medium. After 6 hours, cells were treated with nocodazole as indicated (Table 1). To measure the mitotic index, cells were then trypsinized and transferred to slides by cytospinning at 500 rpm for 6 min. Cells were stained with 4′,6-diamidino-2-phenylindole (DAPI) and donkey antibody to rabbit IgG (*14*). IgG$^+$ cells and IgG$^+$ mitotic cells were counted by immunofluorescence microscopy (*14*).
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14. Cells were fixed at −20°C with 100% methanol for 6 min and permeabilized at −20°C with 100% acetone for 30 s. After being blocked in PBS containing 3% bovine serum albumin, cells were stained with affinity-purified anti-hsMAD2 (2 μg/ml) in the blocking buffer for 1 hour. Cells were then washed six times with PBS containing 0.1% Triton X-100 and incubated for 30 min with donkey antibody to rabbit IgG conjugated to fluorescein isothiocyanate (FITC) (1:50 dilution, Amersham). After six washes in PBS, cells were stained with DAPI (0.1 μg/ml in PBS) and mounted. For coimmunostaining of hsMAD2 and centromeres, cells were incubated with both anti-hsMAD2 and human antibody to centromeres (1:100 dilution) for 1 hour, washed as described above, and incubated with donkey antibody to rabbit IgG conjugated to FITC and donkey antibody to human IgG labeled with rhodamine (both at 1:50 dilution, Jackson ImmunoResearch).
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19. We thank R. Davis for the *cbf1* null strain, J. Colicelli for the human cDNA library, K. Elkon for the human autoantibody to centromeres, A. Murray for communicating unpublished results, and J. Roberts for critical reading of the manuscript. Y.L. is a Jack and Susan Rudin Scholar in the Biomedical Sciences. Supported by an NSF grant (IBN-9118977) to R.B. and a core National Cancer Institute grant (P30-CA-08748).

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