Up-regulation of the mitotic checkpoint component Mad1 causes chromosomal instability and resistance to microtubule poisons

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The mitotic checkpoint is the major cell cycle checkpoint acting during mitosis to prevent aneuploidy and chromosomal instability, which are hallmarks of tumor cells. Reduced expression of the mitotic checkpoint component Mad1 causes aneuploidy and promotes tumors in mice [Iwanaga Y, et al. (2007) Cancer Res 67:160–166]. However, the prevalence and consequences of Mad1 overexpression are currently unclear. Here we show that Mad1 is frequently overexpressed in human cancers and that Mad1 up-regulation is a marker of poor prognosis. Overexpression of Mad1 causes aneuploidy and chromosomal instability through weakening mitotic checkpoint signaling caused by mislocalization of the Mad1 binding partner Mad2. Cells overexpressing Mad1 are resistant to microtubule poisons, including currently used chemotherapeutic agents. These results suggest that levels of Mad1 must be tightly regulated to prevent aneuploidy and transformation and that Mad1 up-regulation may promote tumors and cause resistance to current therapies.

Aneuploidy, an abnormal chromosome number that deviates from a multiple of the haploid, is a common characteristic of tumor cells, occurring in ∼85% of all human cancers (1). Some aneuploid cells also exhibit chromosomal instability (CIN), and have constantly evolving karyotypes. The mitotic checkpoint, also known as the spindle assembly checkpoint, is the major cell cycle checkpoint acting during mitosis to prevent aneuploidy and CIN (reviewed in refs. 2, 3). This checkpoint delays separation of the replicated chromosomes until each and every chromosome has made stable attachments to the microtubules of the mitotic spindle (4–6). This promotes accurate chromosome segregation and the production of genetically identical progeny. Reduction of individual components of the mitotic checkpoint, including Mad1, Mad2, Bub1, BubR1, Bub3, CENP-E, and Mps1, leads to aneuploidy and CIN in multiple systems (7–20).

The identification of mutations in two mitotic checkpoint genes in colorectal cancer cell lines suggested a possible mechanism for CIN in human tumors (21). However, extensive analysis has revealed that mitotic checkpoint genes are only rarely mutated in human cancers. Reduced expression is more common, though effects of reduction of many mitotic checkpoint components on mitosis, the mitotic checkpoint, aneuploidy, and transformation remain to current therapies.
Right). Fifteen of 25 tumor samples (60%) expressed Mad1 at levels greater than fivefold more than those in normal samples, whereas three of 25 tumors (12%) had Mad1 levels <0.5 fold that of normal samples (Fig. 1B). The remaining seven tumors (28%) had levels similar to normal samples (0.5 < n < 5). Overexpression of Mad1 was substantial, with 11 of 25 cancers (44%) having Mad1 expression levels >35 fold greater than those of normal controls. The highest-expressing tumors had a Mad1 expression level >100 fold greater than the median level of expression in noncancerous controls (Fig. 1B). High-expressing cells often exhibited Mad1 puncta (Fig. 1A, Right, Inset), similar to those reported previously in cells transiently overexpressing Mad1 to be annulate lamellae, storage compartments for nuclear pore components (34).

Expression of some mitotic checkpoint components is elevated during mitosis, making it difficult to determine whether increased expression in tumors is simply a result of a higher rate of proliferation. However, we do not believe this is the reason for increased expression of Mad1 in tumors for several reasons. First, Mad1 is expressed throughout the cell cycle, making it unlikely that protein levels vary 35 to 100 fold during a typical cell cycle. Second, the Mad1 promoter has been reported to be predominantly active during G1, suggesting that Mad1 protein levels could be expected to be reduced in cells with a high mitotic index (50). However, as assessed by immunoblotting, Mad1 protein levels do not exhibit cell cycle regulated expression in yeast (51) or human (34). Consistent with this, Mad1 fluorescence intensity does not vary more than twofold from its average value in cells determined to be in G1, S, or G2 by DAPI fluorescence intensity (Fig. 1C and D). Finally, none of the tumors in the microarray had a mitotic index greater than 1.8%. We conclude that primary cancers frequently overexpress Mad1 protein and that this is not simply caused by an increased rate of proliferation.

**Mad1 Overexpression Is a Marker of Poor Prognosis.** To determine the relevance of Mad1 overexpression in human tumors, we screened a microarray dataset containing 242 breast tumors from the Stockholm and Uppsala cohorts described by Ishii et al. (52) [Gene Expression Omnibus (GEO) accession no. GSE4922]. Mad1 expression level in the tumors was classified as high, low, or intermediate if Mad1 expression was twofold higher, lower, or within twofold of the median expression level in the tumors, respectively. Patients displayed a Mad1 expression dependent decrease in 12-y survival. Patients with high Mad1 expression had only a 33% survival rate at 12 y, whereas patients with intermediate levels of Mad1 had a 65% survival rate, and patients with low expression of Mad1 displayed greater than 80% survival (Fig. 1E). Mad1 status showed a positive correlation with lymph node involvement, tumor size, and grade (SI Appendix, Table S1). Interestingly, there is a significant correlation between Mad1 expression levels and p53 status in this cohort such that Mad1-high tumors were more likely to be p53 mutant than Mad1-low tumors. There was no correlation between Mad1 expression levels and estrogen receptor status, a key determinant of patient outcome. Thus, Mad1 overexpression is a marker of poor prognosis in breast cancer that is independent of hormone status.

**Up-Regulation of Mad1 Causes Aneuploidy.** Well-established and characterized breast cancer cell lines (e.g., MCF-7, MDA-MB-231, T47D, SK-BR-3) are extensively aneuploid, complicating studies of chromosome segregation. Interestingly, Mad1 expression has been reported to be up-regulated in colon adenocarcinomas compared with colon adenomas (GEO accession no. GSE8067) (49) and well-characterized chromosomally stable
colonic cancer cell lines do exist. To determine if Mad1 overexpression is also a marker for poor prognosis in colon cancer, we screened a microarray dataset containing 83 stage II and III colorectal cancers from the cohort described by Sweeney et al. (53) (GEO accession no. GSE24549). Patients with high Mad1 expression had only a 37% survival rate at 10 years, whereas patients expressing low levels of Mad1 had a 62% survival rate (SI Appendix, Fig. S1A). For these reasons, we determined the effects of Mad1 overexpression in a chromosomally stable colorectal cancer cell line (DLD1).

DLD1 cells stably expressing tet-inducible Mad1 or Mad1-YFP were generated. Addition of tetracycline induced expression of Mad1-YFP in a dose-dependent manner (Fig. 2A). A single administration of tetracycline was sufficient to induce Mad1-YFP protein for at least 4 days (SI Appendix, Fig. S1B). The level of overexpression was measured by immunoblotting serially diluted extracts (SI Appendix, Fig. S1 C and D) and by quantitative immunofluorescence (SI Appendix, Fig. S1 E and F), which gave similar results. A dose of tetracycline that induced Mad1-YFP (SI Appendix, Fig. S1C) or untagged Mad1 (SI Appendix, Fig. S1D) to a level equivalent to the median fold change of Mad1 overexpression in human tumors (Fig. 1 A and B) was chosen for further analysis.

Like endogenous Mad1 (SI Appendix, Fig. S1E), Mad1-YFP localized to the nucleus and nuclear envelope in interphase cells (Fig. 2B) and to kinetochores during mitosis (Fig. 2C). Kinetochoore localization was confirmed by colocalization with CENP-E (Fig. 2C). Expression of untagged Mad1 resulted in a similar localization pattern during interphase (SI Appendix, Fig. S1E) and mitosis (SI Appendix, Fig. S1 G–I). Although expression of Mad1 was elevated, kinetochore recruitment was not substantially increased (SI Appendix, Fig. S1 H and I). In addition to exhibiting the expected localization pattern, overexpressed Mad1 and Mad1-YFP apparently saturated the available binding sites and localized to additional sites as well (Fig. 2 B and C, arrows, and SI Appendix, Fig. S1E), as seen in the tumor microarray (Fig. 1A, Right). These sites were previously identified as annulate lamellae, storage compartments for NPC proteins, on the basis of their colocalization with nuclear pore components (34). Consistent with this, overexpressed untagged Mad1 partially colocalized at presumptive annulate lamellae and at the nuclear envelope with the mAb414 antibody, which recognizes the FG repeats found in multiple NPC proteins (SI Appendix, Fig. S1E).

To determine the effect of Mad1 overexpression on ploidy, chromosome number was determined in chromosome spreads (also known as metaphase spreads; Fig. 2D) from cells with or without induction of Mad1-YFP. Control DLD1 cells were predominantly diploid, whereas cells overexpressing Mad1-YFP exhibited significant aneuploidy (Fig. 2E). Examination of the number of chromosomes per cell identified frequent gain and loss of small numbers of chromosomes with little evidence of tetraploidy (Fig. 2F). Similar results were obtained in cells overexpressing untagged Mad1 (SI Appendix, Fig. S2 A–C).

**Fig. 2.** Up-regulation of Mad1 causes aneuploidy. (A) Tet-inducible expression of Mad1-YFP. Coomassie staining was used as a loading control. (B) Mad1-YFP (green) localizes to nuclei in interphase, like endogenous Mad1. Arrows indicate that Mad1-YFP also localizes to additional sites previously identified as annulate lamellae (34). (C) Mad1-YFP (green) localizes to kinetochores (marked with CENP-E, red) during mitosis, as well as to additional sites (arrows). (D) Chromosome spread of DLD1 cell. (E) DLD1 cells expressing Mad1-YFP for 48 h have higher levels of aneuploidy than control cells (n = 100 cells from two independent experiments; *P < 0.05, t test). (F) Chromosome numbers in control and Mad1-YFP-expressing cells show near diploid aneuploidy with minimal tetraploidy (n = 100 cells from two independent experiments; SI Appendix, Fig. S2 A–C, shows results with untagged Mad1).
frequency of lagging chromosomes in anaphase and telophase (Fig. 3 A–D; note white arrows in Fig. 3 B and C). Polar chromosomes, which remained near one of the spindle poles in anaphase and appeared to be misaligned upon chromosome disjunction, were only rarely detected (Fig. 3 C and D, yellow arrow). Similar results were obtained when examining cells overexpressing untagged Mad1 (SI Appendix, Fig. S2 D and E).

To observe chromosome segregation defects more closely, cells stably expressing histone H2B-RFP were observed by live cell microscopy. Control cells exhibited predominantly normal divisions in which they waited for the last chromosome to align before segregating their chromosomes equally to produce genetically identical daughters (Fig. 3 E and F and Movie S1). Cells overexpressing Mad1 exhibited a variety of phenotypes of differing severity. Approximately one quarter (24.1%) of cells overexpressing Mad1 had lagging chromosomes in anaphase and telophase with no other observable defects (Fig. 3 A). Approximately 46% of control cells completing anaphase had chromosomes that had not yet segregated to opposite poles (Fig. 3 B). Most chromosomes that were not segregated totally disjoined and were only rarely detected (Fig. 3 C and D). In addition to cells with lagging chromosomes, time-lapse analysis revealed a population of Mad1-overexpressing cells (17.1%) that entered anaphase in the presence of misaligned chromosomes (Fig. 3 E and H and Movie S3). Another 17.2% entered anaphase with misaligned chromosomes and exhibited lagging chromosomes as well (Fig. 3 E and I and Movie S4), bringing the percentage of cells with lagging chromosomes in anaphase and telophase to 41.4%, similar to that observed in our fixed-cell analysis. All told, 58.6% of Mad1-overexpressing cells missegregated chromosomes during mitosis, consistent with a CIN phenotype (Fig. 3 E).

Overexpression of Mad1 Disrupts Mitotic Timing. Ectopic expression of Mad2 has been shown to delay the metaphase-to-anaphase transition (S4). To determine whether overexpression of Mad1 had a similar or different effect, the timing of mitosis was determined by phase-contrast microscopy. Whereas control cells elongated in an average of 47.7 min and completed mitosis (scored as the time from rounding to flattening) in 109.3 min, cells overexpressing Mad1 elongated in 22.7 min and completed mitosis in 61.8 min (Fig. 4 A–C and Movies S5 and S6). Thus, Mad1-overexpressing cells traversed mitosis in approximately half the time of control cells.

Increased Expression of Mad1 Weakens the Mitotic Checkpoint. The high percentage of cells entering anaphase with misaligned chromosomes, coupled with the reduced mitotic timing, suggested that the mitotic checkpoint is weakened by increased expression of Mad1. As an initial method of examination, the status of the mitotic checkpoint was determined by measuring the mitotic index after challenge with microtubule poisons. Mitotic index was measured in live cells treated with the DNA-binding dye Hoechst 33258 by phase-contrast and fluorescence microscopy (Fig. 4D). As expected, control cells accumulated in mitosis in response to loss of microtubules caused by treatment with colcemid, with the maximal mitotic index occurring 20 h after drug addition (Fig. 4E). However, cells expressing Mad1-YFP exhibited a maximal mitotic index approximately fourfold lower (Fig. 4E). A similar failure to accumulate in mitosis in response to colcemid was observed in cells overexpressing untagged Mad1 (Fig. 4F). Importantly, Mad1-overexpressing cells also failed to accumulate in mitosis in response to the clinically useful microtubule poisons paclitaxel (Taxol) and vinblastine (vinbl). These drugs represent microtubule poisons with different mechanisms, as paclitaxel stabilizes microtubules, whereas vinblastine results in net loss of spindle microtubules, similar to colcemid (Fig. 4F). To determine whether this effect was unique to DLD1 cells, we tested the effect of Mad1-YFP expression on the mitotic checkpoint in HeLa cells. Like DLD1 cells, HeLa cells stably expressing Mad1-YFP showed a significantly decreased mitotic index in response to colcemid, paclitaxel, and vinblastine (Fig. 4G).

Defects in mitotic checkpoint signaling are sufficient to cause chromosome missegregation (9, 14, 19). However, multiple mitotic checkpoint components, including Bub1, BubR1, and CENP-E, also function in chromosome congression (13, 55–58). To determine whether overexpression of Mad1 inhibited congression, DLD1 cells were treated with the proteasome inhibitor MG132 to prevent sister chromatid disjunction and accumulate cells in metaphase. Cells overexpressing untagged Mad1 aligned their chromosomes to form metaphase plates at a rate indistinguishable from control cells (SI Appendix, Fig. S2F), suggesting that overexpression of Mad1 does not cause defects in congression. To further examine chromosome alignment in cells up-regulating Mad1, we treated cells expressing histone H2B-RFP and GFP-Tubulin with monastrol to induce monopolar spindles. The monastrol was then washed out and replaced with MG132. Spindle assembly and chromosome congression after...
monastrol washout were observed by time-lapse microscopy. Compared with controls, cells expressing elevated levels of Mad1 were not delayed in aligning chromosomes under these conditions (*SI Appendix, Fig. S2G*).

Congression defects are often associated with a decrease in kinetochore-microtubule attachments, which can be detected by a reduction in interkinetochore distance (13, 17, 56, 59, 60). As a further test of whether Mad1 overexpression negatively influenced kinetochore-microtubule attachments, we measured the interkinetochore distance in control cells and those that overexpress untagged Mad1. No difference in interkinetochore distance was observed in Mad1-overexpressing cells (1.11 ± 0.16 μm; n = 139) compared with control cells (1.13 ± 0.18 μm; n = 134; P = 0.3245; *SI Appendix, Fig. S2 H and I*). This is consistent with recent data showing no role for Mad1 in kinetochore-microtubule turnover, at least when Mad1 is depleted by siRNA (61). Thus, the chromosome missegregation phenotype in Mad1-overexpressing cells appears to occur as a result of a deficit in mitotic checkpoint signaling coupled with a decrease in the duration of mitosis, as opposed to defects in kinetochore–microtubule interactions.

**Overexpressed Mad1 Mislocalizes Mad2 from Kinetochore.** To determine the mechanism underlying the deficit in mitotic checkpoint signaling, kinetochore recruitment of several mitotic checkpoint components was determined. Overexpression of Mad1 did not affect kinetochore localization of Bub1, BubR1, or CENP-E (Fig. 5 A and B). Although a portion of the Mad1 binding partner Mad2 remained at kinetochores (*SI Appendix, Fig. S1F*), quantitative immunofluorescence revealed that the majority of Mad2 was mislocalized from kinetochores (Fig. 5 A and B) and colocalized with overexpressed Mad1 at non-chromosomal sites (*SI Appendix, Fig. S2A*). Mad2 kinetochore recruitment was reduced to approximately one third the level in control cells (Fig. 5B). Similar results were observed in an independent clone of cells overexpressing Mad1 (*SI Appendix, Fig. S3B*).

Removal of Mad2 from kinetochores was not caused by loss of Mad2 protein, as Mad2, BubR1, Bub1, and CENP-E are all expressed at equivalent levels in Mad1-YFP–expressing cells as compared with controls (Fig. 5C). Mad1-YFP–expressing cells also showed reduction of Mad2 at kinetochores (Fig. 5 D and E) and relocalization of Mad1 with overexpressed Mad1-YFP (*SI Appendix, Fig. S3C*).

Normally, Mad2 is expressed in molar excess over Mad1 (35, 38, 62). Consistent with this, in control DLD1 cells, all detectable Mad1 is immunoprecipitated with Mad2 (*SI Appendix, Fig. S3D*, lanes 7 and 8) whereas only a fraction of detectable Mad2 immunoprecipitates with Mad1 (*SI Appendix, Fig. S3D*, lanes 3 and 4). However, in Mad1-overexpressing cells, nearly all Mad2 is immunoprecipitated with Mad1 (*SI Appendix, Fig. S3D*, lanes 5 and 6) whereas a portion of Mad1 remains soluble after immu-

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**Fig. 5.** Mad2 is mislocalized from kinetochores in cells expressing excess Mad1. (A) Quantitative immunofluorescence of Mad2, BubR1, Bub1, CENP-E (green), and DNA (blue) in cells treated with colcemid for 1 h. (Scale bar, 2.5 μm.) (B) Quantification of kinetochore fluorescence intensity (n = 37–119 cells from at least three independent experiments; *P < 0.05, t test). *SI Appendix, Fig. S3B*, shows quantification of an independent clone of Mad1-overexpressing cells. (C) Mad1-YFP expression does not affect expression levels of other mitotic checkpoint components including BubR1, Bub1, Mad2, and CENP-E. Coomassie stain was used as a loading control. (D) Mad2 localizes to kinetochores during mitosis in control cells, but is largely mislocalized in cells expressing Mad1-YFP. (Scale bar, 5 μm.) (E) Quantification of Mad2 kinetochore fluorescence in Mad1-YFP–expressing cells (n > 32 cells from three independent experiments; **P < 0.001, t test).
noprecipitation of Mad2 (SI Appendix, Fig. S3D, lanes 9 and 10). Thus, the pool of free Mad2 that is not bound to Mad1 is diminished in Mad1-overexpressing cells.

**Overexpression of Mad1 Enhances Transformation.** Up-regulation of Mad1 or Mad1-YFP reduced population growth rates compared with control cells (Fig. 6A and B). This proliferative disadvantage was coupled with an increase in cell death (Fig. 6C), consistent with recent reports that clonal survival is inhibited in chromosomally stable cancer cell lines forced to exhibit CIN (63, 64). Interestingly, the surviving cells are significantly more likely to exhibit anchorage independent growth in soft agar, a marker of transformation. Although control cells exhibited limited growth in soft agar (Fig. 6D, Left, and Fig. 6E), ectopic expression of Mad1-YFP caused a 3.4-fold increase in the number of anchorage-independent colonies (Fig. 6D, Right, and Fig. 6E). Similar results were observed in cells overexpressing untaged Mad1 (SI Appendix, Fig. S3 E and F). This is reminiscent of a previous report that overexpression of Mad2 inhibits growth of cells in culture, but drives tumorigenesis in mice (65).

If aneuploidy has a role in driving anchorage independent growth, colonies from Mad1-overexpressing cells should be more aneuploid than colonies from parental cells. To test this, we scored soft agar colonies for aneuploidy by using chromosome spreads. Three colonies each of control and Mad1-overexpressing cells were examined. In all cases, Mad1-overexpressing cells were significantly more aneuploid than controls (Fig. 6F), consistent with the hypothesis that aneuploid cells contribute to anchorage independent growth.

One potential concern is that, to date, the limited number of CIN cancer cell lines tested for mitotic checkpoint activity based on their ability to prevent anaphase onset in the presence of misaligned chromosomes (HT29, Caco2, MCF-7, SW480, and SW837) have all had an intact mitotic checkpoint (63, 66). Instead, these cells exhibit CIN as a result of lagging chromosomes likely caused by merotelic attachments. However, this does not demonstrate that mitotic checkpoint defects do not occur in any cancer cells or cell lines. Indeed, MDA-MB-231 breast cancer cells exhibit a weakened mitotic checkpoint. Eight of 49 (16%) MDA-MB-231 cells with bipolar spindles that expressed histone H2B-RFP and GFP-tubulin entered anaphase with misaligned chromosomes when examined by time-lapse microscopy (SI Appendix, Fig. S4 and Movie S7).

**Cells Expressing High Levels of Mad1 Are Resistant to Microtubule Poisons.** The role of the mitotic checkpoint in the response to drugs that cause mitotic arrest remains controversial (67). Multiple groups have reported that a functional mitotic checkpoint is required for sensitivity to microtubule poisons and that a weakened mitotic checkpoint confers resistance (68–71). However, numerous other groups have found that cells with a weakened mitotic checkpoint are more sensitive to microtubule poisons (72–74) or that there is no difference (66, 75, 76). One recent hypothesis is that mitotic checkpoint impairment causes re-
sistance to microtubule poisons in the short term (48–72 h), but does not confer resistance in longer-term studies (4–11 d) (75).

We tested the sensitivity of cells expressing increased levels of Mad1 to microtubule poisons at both short and long time points. Interestingly, up-regulation of Mad1 caused resistance to microtubule poisons after 72 h and 10 d of treatment. Death was determined based on DNA and cellular morphology by using phase-contrast and fluorescence microscopy of live cells treated with Hoechst 33258 (Fig. 7A). At 72 h after treatment with the microtubule poison colcemid, 36.5 ± 0.8% of control cells exhibited cell death, as opposed to 1.8 ± 0.1% of cells expressing Mad1-YFP (Fig. 7B). Overexpression of untagged Mad1 yielded similar results, with 45% to 65% death in control cells treated with colcemid, vinblastine and paclitaxel, but only 15% to 18% death in cells expressing increased levels of Mad1 (Fig. 7C).

To determine whether up-regulation of Mad1 merely delayed cell death, cell number was determined over a 10-d exposure to colcemid. Cells expressing Mad1-YFP were significantly more viable than control cells over the time course (Fig. 7D). Similarly, cells expressing elevated levels of Mad1 were significantly more viable after 10 d of treatment with colcemid, paclitaxel and vinblastine (Fig. 7E). Mad1-overexpressing cells remained viable 1 wk after drug washout (SI Appendix, Fig. S5A), and proliferated into colonies when plated at low density (SI Appendix, Fig. S5B).

To better understand the short-term effects of Mad1 up-regulation on long-term mitotic arrest, cells were observed by time-lapse microscopy for ≥65 h after addition of microtubule poison. Cells expressing elevated levels of Mad1 were significantly less likely than controls to die from mitosis and were significantly more likely to slip out of mitosis (Fig. 7F). Of cells that slipped out of mitosis to form tetraploid G1 cells, most cells overexpressing Mad1 (75%) survived the duration of the movie, at least 30 h after slippage, whereas most control cells that slipped (64%) died during the period of observation (Fig. 7G).

To rule out the possibility that this effect of Mad1 up-regulation is unique to DLD1 cells, HeLa cells stably expressing Mad1-YFP were tested for their response to colcemid, paclitaxel and vinblastine. As in DLD1 cells, expression of Mad1-YFP in HeLa cells led to reduced cell death after treatment with microtubule poisons (Fig. 7H).

Based on the effects of Mad1 overexpression on mitosis, it is likely that Mad1 up-regulation causes resistance to microtubule poisons by reducing mitotic checkpoint activity. The effects on mitosis would not be predicted to affect the response to DNA damaging agents. To test this, rates of cell death and cell survival were measured after treating DLD1 cells with the topoisomerase II poisons doxorubicin (Adriamycin) and VP16 (etoposide). Cell death and survival rates in Mad1-overexpressing cells were indistinguishable from rates in control cells (Fig. 7 I and J). Thus, up-regulation of Mad1 decreases sensitivity to microtubule poisons currently used to treat human cancers without affecting the response to DNA damaging agents.

**Discussion**

Our results are consistent with a model in which up-regulation of Mad1 weakens mitotic checkpoint signaling by titrating the soluble pool of Mad2 (Fig. 8). Normally, endogenous Mad2 is expressed in excess of endogenous Mad1 (35, 38, 62), permitting a soluble pool of Mad2. This soluble pool is converted into active inhibitors of APC-Cdc20 after transiently binding Mad1–Mad2 heterodimers at unattached kinetochores (Fig. 8A) (36). DLD1 cells that overexpress Mad1 to a level equivalent to the median extracts requires both Mad1-bound and Mad1-free Mad2 (62).
Multiple mitotic checkpoint proteins, including Bub1, BubR1, and CENP-E, have been shown to independently participate in chromosome congression (13, 55–58). However, we find that Mad1-overexpressing cells do not have defects in chromosome alignment or in kinetochore-microtubule attachments. This is consistent with overexpression of Mad1 weakening mitotic checkpoint signaling by titrating Mad2, as Mad2-depleted cells do not exhibit defects in congression, and missegregate chromosomes as a result of mitotic checkpoint defects coupled with a reduction in the duration of mitosis (13, 70, 77).

It has recently been shown that CIN cancer cell lines do not necessarily have weakened mitotic checkpoints (63, 66, 78). However, we show here that weakening of the mitotic checkpoint as a result of up-regulation of Mad1 does cause CIN. We also show a CIN cancer cell line with a mitotic checkpoint deficit. Thus, the status of the mitotic checkpoint in a given cell line cannot be predicted merely based on CIN, and must be determined empirically.

Mice expressing reduced levels of Mad1 develop spontaneous and carcinogen-induced tumors with increased frequency (10). The tumor microarray datasets with patient outcome we analyzed contained only tumor samples. Thus, we were unable to compare Mad1 levels in these tumors with Mad1 levels in control tissue. It is unclear whether patients with low Mad1 and increased survival express lower levels of Mad1 than healthy controls. However, mounting evidence suggests that overexpression of mitotic checkpoint components may be more detrimental, and more physiologically relevant, than underexpression. Multiple mitotic checkpoint components, including Mad1, Mad2, and Bub1, are frequently up-regulated in human cancers (43, 79, 80). Indeed, more than half of the breast cancers in our study expressed Mad1 protein at levels more than fivefold those in control breast samples (Fig. 1 A and B). Mouse models overexpressing the mitotic checkpoint components Mad2 (65) and Bub1 (80) have now been reported. Overexpressing Mad2 is significantly more tumorigenic than reducing its expression, and produces tumors with shorter latencies and higher penetrance (14, 65). Similarly, mice overexpressing Bub1 develop tumors with higher frequency and shorter latencies than mice with reduced levels of Bub1 (11, 80). Here we show that up-regulation of Mad1 is prevalent in human tumors with poor outcomes and that overexpression of Mad1 affects cell cycle progression in culture. Together, this suggests that up-regulation of mitotic checkpoint components, rather than down-regulation, may be a more clinically relevant mechanism of promoting transformation and tumorigenesis.

Microtubule poisons are currently used to treat a host of cancers, including those of the lung, breast, ovaries, and testes. However, a substantial number of patients do not benefit from their use. Currently, there are no biomarkers available to predict patient response to these drugs. A clinically useful biomarker to predict response could significantly improve patient outcomes while simultaneously decreasing serious side effects and treatment costs. As excess Mad1 confers resistance to the clinically relevant microtubule poisons paclitaxel and vinblastine, up-regulation of Mad1 may have therapeutic relevance as a predictive biomarker.

### Immunofluorescence and Immunoblotting

For immunofluorescence, cells were washed with MicroTubule Stabilizing Buffer (MTSB; 100 mM K-Pipes, pH 6.8, 0.5 mM MgCl2, 1 mM EGTA) and 0.05% Triton X-100 in MTSB. Cells were fixed with warm 0.5% Triton X-100 in MTSB at 37 °C, and fixed with warm 4% formaldehyde in MTSB. Cells were blocked in Triton Block (2.5% (vol/vol) FBS, 200 mM glycerine, and 0.1% Triton X-100 in PBS solution) overnight at 4 °C. Antibodies were diluted in Triton Block. DNA was detected with DAPI, and cells were mounted with Vectashield. Staining was performed with antibodies to Mad1 (1:100 or newly generated, as detailed later; gift of A. Musacchio, IONC, Rome, Italy), Mad2 (1:200; B1), CENP-E (Hpx; 1:200) (82), BubR1 (5F9; 1:200) (83), and Bub1 (1:200). YFP was visualized directly.

Images were acquired on a Nikon Ti-E inverted microscope using a CoolSNAPHQ2 camera driven by Nikon Elements software. Chromosome spread images are single z-planes acquired using a 100×-oil immersion objective. Staining was performed on 3D deconvolved images shown in Fig. 5 A and B. Quantiﬁcation shown in Fig. 5 C was performed on 3D z-stacks by using the volume measurement tool in Elements. For analysis of Mad1 and Mad2, quantiﬁcation was performed on maximum projections in areas identiﬁed as kinetochores by localization of BubR1 (for Mad2) or Bub1 (for Mad1). The intensity measure was the mean intensity of Mad1 or Mad2 at Bub1 or Bub1-positive kinetochores.

### Immunohistochemistry

Five-micrometer sections of formalin-ﬁxed, parafﬁn-embedded tissue microarrays (gift of A. Friedl, University of Wisconsin, Madison, WI) were subjected to antigen retrieval in citrate buffer, serum-free retrieval solution by microwave heat. Sections with 100 ng/mL puromycin, 50 mM Hepes, pH 7, 5 mM EDTA) and 5x sample buffer. Proteins were separated by SDS/PAGE, transferred to nitrocellulose, and probed with antibodies at the same concentrations used for immunofluorescence.

**Materials and Methods**

**Cell Culture.** DLD1 colorectal cancer cells were grown in DMEM supplemented with 10% (vol/vol) FBS, 2 mM L-glutamine, and 50 μg/mL penicillin/streptomycin at 37 °C and 5% CO2. Cells were selected with 400 μg/mL puromycin. Single clones were isolated by using a4 0 μL of cells were dropped onto preextracted for 5 min with warm 0.5% Triton X-100 in MTSB at 37 °C, and fixed with warm 4% formaldehyde in MTSB. Cells were blocked in Triton Block (2.5% (vol/vol) FBS, 200 mM glycerine, and 0.1% Triton X-100 in PBS solution) overnight at 4 °C. Antibodies were diluted in Triton Block. DNA was detected with DAPI, and cells were mounted with Vectashield. Staining was performed with antibodies to Mad1 (1:100 or newly generated, as detailed later; gift of A. Musacchio, IONC, Rome, Italy), Mad2 (1:200; B1), CENP-E (Hpx; 1:200) (82), BubR1 (5F9; 1:200) (83), and Bub1 (1:200). YFP was visualized directly.

Images were acquired on a Nikon Ti-E inverted microscope using a CoolSNAPHQ2 camera driven by Nikon Elements software. Chromosome spread images are single z-planes acquired using a 100×-oil immersion objective (NA 1.4). All other images of tissue culture cells are from 0.2 μm z-stacks collected using a 100×-oil immersion objective. Chromosome spreads were deconvolved by using the AQI 3D Deconvolution module in Elements. Unless otherwise specified, 2D maximum projections are shown. Maximum projections were assembled in Elements. Overlays were generated in Photoshop. Quantitative imaging was performed using identical exposure times on samples prepared identically and imaged in a single sitting. With the exception of the Mad1 and Mad2 quantification shown in Fig. 5 A and B, quantification was performed on 3D z-stacks by using the volume measurement tool in Elements. For analysis of Mad1 and Mad2, quantification was performed on maximum projections in areas identified as kinetochores by localization of BubR1 (for Mad2) or Bub1 (for Mad1). The fluorescence intensity of Mad1 and Mad2 at kinetochores was calculated by subtracting the average of the background signal in the four quadrants surrounding the kinetochore from the mean intensity of Mad1 or Mad2 at Bub1 or Bub1-positive kinetochores.

For immunoblotting, equal numbers of cells were resuspended in ELB lysis buffer (250 mM NaCl, 0.1% Nonidet P-40, 50 mM Hepes, pH 7, 5 mM EDTA) and 5x sample buffer. Proteins were separated by SDS/PAGE, transferred to nitrocellulose, and probed with antibodies at the same concentrations used for immunofluorescence.

**Immunohistochemistry.** Five-micrometer sections of a formalin-fixed, paraffin-embedded tissue microarray (gift of A. Friedl, University of Wisconsin, Madison, WI) were subjected to antigen retrieval in citrate buffer, serum-blocked, and stained with rabbit anti-Mad1 antibody (described in the following paragraph), a mixture of e-cadherin and cytokeratin antibodies to identify epithelial cells (Dako), and DAPI overnight at 4 °C. Alexa Fluor-conjugated secondary antibodies were used. Images were acquired on a Nikon Ti-E inverted microscope by using a CoolSNAPHQ2 camera driven by Nikon Elements software. Images were acquired using identical exposure times in a single imaging session. Z-stacks (0.2 μm) were collected by using a 40× dry objective (0.75 NA) and deconvolved by using the AQI 3D Deconvolution module in Elements. Maximum projections were quantified in Elements. DAPI signal was used to set a threshold defining a binary mask including the nucleus and perinuclear region to ensure inclusion of nuclear envelope staining. This binary mask was converted to a region of interest in which all Mad1 signal was quantified. Quantification was based on the modal pixel intensity in all DAPI-positive regions following a background...
A column of Mad1 amino acids 329 to 621 following removal of the GST tag


A heated chamber and an automated stage at 37 °C. Images were acquired

with sterile mineral oil. Five

H2B-RFP with or without GFP-tubulin grown in 35-mm dishes with glass


To measure mitotic timing, cells were grown in CO2-independent media

To determine the aggressiveness of tumors. Expression data were log2-transformed

The authors thank Dr. A. Friedl, Dr. A. Musacchio, and Dr. T. Yen for sharing tumor microarray slides, Mad1 antibody, and a mouse model, respectively. This work was supported in part by National Institutes of Health Grants 1R01CA140458 (to B.A.W.), GM088151 (to A.A.) and T32 GM008688 (E.M.C.B. and L.M.Z.) and American Cancer Society Grant IRG-58-011-48 (to B.A.W.).

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Supporting Information

Up-regulation of the mitotic checkpoint component Mad1 causes chromosomal instability and resistance to microtubule poisons

Ryan et al, 2012

Supporting Information Inventory

Figure S1. Inducible expression of Mad1 and Mad1-YFP.

Figure S2. Overexpression of Mad1 results in aneuploidy and chromosomal instability.

Figure S3. Upregulation of Mad1 mislocalizes Mad2 from kinetochores and enhances transformation.

Figure S4. A weakened mitotic checkpoint in human cancer cells.

Figure S5. Long-term viability of Mad1-overexpressing cells treated with microtubule poisons.

Table S1. Mad1 status positively correlates with lymph node involvement, tumor size and grade, but is independent of hormone status.
Figure S1. Inducible expression of Mad1 and Mad1-YFP. (A) Colorectal cancer patients with tumors expressing high levels of Mad1 have lower 10 year survival rates than patients with low expression of Mad1. (B) A single administration of tetracycline results in expression of Mad1-YFP protein for ≥4 days. (C-D) 0.25 µg/mL tetracycline
results in expression of Mad1-YFP (C) and untagged Mad1 (D) to a level similar to the median level of Mad1 overexpression in human cancers (~50-fold; Fig. 1A, B). (E) Overexpressed Mad1 localizes to nuclei, the nuclear envelope, and annulate lamellae. Control and Mad1 overexpressing cells were costained with antibodies to Mad1 and mAb414, which recognizes the FG repeats found in multiple nuclear pore components. Left panels, maximum projections showing that endogenous Mad1 localizes to the nucleus and nuclear envelope in control cells. Center, single z plane of control cell highlighting the nuclear envelope localization of Mad1. Right, single z plane of cell overexpressing untagged Mad1. Arrows show colocalization of mAb414 and upregulated Mad1 at additional sites previously identified as annulate lamellae (Campbell MS, Chan GK and Yen TJ (2001) J Cell Sci 114:953-963). Even in a single z plane, the intensity of Mad1 staining at annulate lamellae is sufficient to appear saturated when shown quantitatively using the same LUTs as control cells. Bottom, enlargement of single z plane image with enhanced LUTs showing continued localization of upregulated Mad1 at the nuclear envelope. (F) Quantitation of Mad1 overexpression by immunofluorescence of interphase cells, normalized to expression levels in control cells, showing results consistent with immunoblotting shown in C, D. n > 500 cells. ** = p < 0.001, t test. (G-H) Levels of Mad1 at kinetochores are not substantially altered by Mad1 upregulation. (G) Single z planes of cells showing localization of Mad1 at kinetochores, which are marked with Bub1. (H) Quantitation of Mad1 kinetochore localization. n ≥ 45 cells from three independent experiments. (I) Single z plane image showing that overexpressed, untagged Mad1 and a portion of Mad2 localize to unattached kinetochores.
Figure S2. Overexpression of Mad1 results in aneuploidy and chromosomal instability. (A) Chromosome spread of DLD1 cell. Scale bar, 10 µm. (B) DLD1 cells overexpressing Mad1 for 48 hours have higher levels of aneuploidy than control cells. n = 50 cells from each of three independent experiments. * = p < 0.05, t test. (C) Chromosome numbers in control and Mad1 overexpressing cells showing that Mad1 upregulation causes near-diploid aneuploidy with minimal tetraploidy. n = 50 cells from each of three independent experiments. (D) Immunofluorescence of anaphase cells from control (left) and Mad1 overexpressing (center, right) cells. Arrows, lagging chromosomes. (E) Quantitation of abnormal anaphases. n > 100 anaphases from two independent experiments. * = p < 0.05, t test. (F) Mad1 overexpressing cells
were treated with MG132 for various times to prevent anaphase and determine whether overexpression of Mad1 interferes with chromosome congression. n > 250 cells from each of two independent experiments. (G) Mad1 overexpressing cells do not exhibit delays in congression. After 24 hours -/+ tetracycline, DLD1 cells expressing histone H2B-RFP and GFP-tubulin were treated with 100 µM monastrol to accumulate cells with monopolar spindles. After 18-20 hours, cells were washed 3 times with PBS and fresh media containing 20 µM MG132 was added. Cells were observed immediately by timelapse analysis. The time required for chromosomes to form a metaphase plate is shown. n = 69 control and 48 Mad1 overexpressing cells from 2 independent experiments. (H) Single z plane image from a deconvolved stack of a DLD1 cell. Kinetochores are marked with the mitotic checkpoint kinase Bub1, which was used to measure interkinetochore distance. Only sister kinetochores in the same plane were measured. Yellow and white arrows indicate pairs of sister kinetochores. (I) Quantitation of interkinetochore distance in control and Mad1 overexpressing cells. Aligned chromosomes under tension were measured in metaphase cells. Interkinetochore distance of chromosomes in the absence of tension was measured in the presence of the microtubule poison colcemid. No difference in interkinetochore distance was observed between control and Mad1 overexpressing cells under either condition. n = 107-145 sister kinetochores from 8-13 cells per condition. p = 0.3245 (no colcemid) and 0.5354 (plus colcemid).
Figure S3. Upregulation of Mad1 mislocalizes Mad2 from kinetochores and enhances transformation. (A) Overexpression of Mad1 (green) largely removes Mad2 (red) from kinetochores. Scale bar, 5 µm. (B) Overexpression of Mad1 mislocalizes Mad2 but not BubR1, Bub1 or CENP-E from kinetochores in an independent clone of cells distinct from the one shown in Fig. 5A, B. (C) Expression of Mad1-YFP (green) removes most Mad2 (red) from kinetochores. Scale bar, 6 µm. (D) Immunoprecipitation with GST control, Mad1 or Mad2 antibodies shows that, while only a portion of Mad2 is immunoprecipitated with Mad1 in control cells, overexpression of Mad1 moves the bulk of Mad2 from the unbound supernatant fraction (S) to the bound, pellet (P) fraction. (E) Images of cells grown in soft agar in the absence (left) or presence (right) of tet to induce expression of Mad1. Scale bar, 100 µm. (F) Quantitation of colony formation after 10-12 days of growth in soft agar. * = p <0.05, t test. n = 3 experiments performed in triplicate.
Figure S4. A weakened mitotic checkpoint in human cancer cells. MDA-MB-231 breast cancer cell entering anaphase in the presence of a misaligned chromosome due to a weakened mitotic checkpoint. Still images from timelapse acquisition of histone H2B-RFP (left), GFP-tubulin (center) and the overlay (right) in an unperturbed cell that enters anaphase with a misaligned chromosome. Images were acquired every 2 minutes. The third panel from the top shows anaphase onset and the second panel shows the last frame before anaphase onset. The movie is available as Supplemental Video 7. Scale bar, 5 µm.
Figure S5. Long-term viability of Mad1-overexpressing cells treated with microtubule poisons. (A) Crystal violet stained dishes of control and Mad1 overexpressing DLD1 cells immediately after 72 hours of treatment with microtubule poisons (top) or after 72 hours of treatment followed by drug washout and one week of incubation in normal growth medium (bottom). (B) Crystal violet stained colony forming assays of Mad1 overexpressing cells after 72 hours of treatment with the indicated microtubule poisons.
Table S1. Mad1 status positively correlates with lymph node involvement, tumor size and grade, but is independent of hormone status.

<table>
<thead>
<tr>
<th></th>
<th>Elston Grade</th>
<th>Size</th>
<th>Patient Age</th>
<th>Lymph Node Positive</th>
<th>p53 mutant</th>
<th>Estrogen Receptor Positive</th>
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<tbody>
<tr>
<td>Mad1-high (n=27)</td>
<td>2.25 +/- 0.1220 (0.0175)</td>
<td>26.51 +/- 1.989 (0.0072)</td>
<td>59.36 +/- 2.580 (0.2451)</td>
<td>16/28 (0.0288)</td>
<td>12/28 (0.0424)</td>
<td>22/28 (0.4986)</td>
</tr>
<tr>
<td>Mad1-low (n=30)</td>
<td>1.774 +/- 0.1445 (0.0175)</td>
<td>19.90 +/- 1.605 (0.0072)</td>
<td>62.290 +/- 2.796 (0.2451)</td>
<td>7/28 (0.0288)</td>
<td>5/31 (0.0424)</td>
<td>26/30 (0.4986)</td>
</tr>
</tbody>
</table>

Mean values +/- standard error from the Stockholm and Uppsala breast cancer cohorts described by Ivshina et al (GSE4922 in the Gene Expression Omnibus) ; (p-value). Bold values indicate statistical significance (p < 0.05).
Movie S1. Fluorescence (Left), DIC (Center) and overlaid (Right) images of DLD1 cells stably expressing histone H2B-RFP observed by time-lapse fluorescence and DIC microscopy at 2-min intervals using a 60× 1.4NA objective, as described in Materials and Methods. This movie relates to Fig. 3F. Normal mitosis in control cell. Note that the last chromosome aligns before the cell enters anaphase.

Movie S2. Fluorescence (Left), DIC (Center) and overlaid (Right) images of DLD1 cells stably expressing histone H2B-RFP observed by time-lapse fluorescence and DIC microscopy at 2-min intervals using a 60× 1.4NA objective, as described in Materials and Methods. This movie relates to Fig. 3G. Lagging chromosomes in a cell overexpressing Mad1 for 24 hours. The lagging chromosomes each form their own micronucleus.
Movie S3. Fluorescence (Top), DIC (Middle) and overlaid (Bottom) images of DLD1 cells stably expressing histone H2B-RFP observed by time-lapse fluorescence and DIC microscopy at 2-min intervals using a 60× 1.4NA objective, as described in Materials and Methods. This movie relates to Fig. 3H. Cells overexpressing Mad1 entering anaphase with misaligned chromosomes. Both mitotic cells pictured enter anaphase with misaligned chromosomes, some of which form micronuclei.

Movie S3

Movie S4. Fluorescence (Left), DIC (Center) and overlaid (Right) images of DLD1 cells stably expressing histone H2B-RFP observed by time-lapse fluorescence and DIC microscopy at 2-min intervals using a 60× 1.4NA objective, as described in Materials and Methods. This movie relates to Fig. 3I. Cell overexpressing Mad1 which enters anaphase with misaligned chromosomes and also exhibits lagging chromosomes.

Movie S4
Movie S5. Phase contrast time-lapse imaging of DLD1 cells acquired at 10-min intervals using a 10x, 0.13NA objective and perfect focus, as described in Materials and Methods. This movie relates to Fig. 4A Upper. Control DLD1 cells dividing. On average, control cells require 109.3 min to complete mitosis (defined as cell rounding to flattening).

Movie S6. Phase contrast time-lapse imaging of DLD1 cells acquired at 10-min intervals using a 10x, 0.13NA objective and perfect focus, as described in Materials and Methods. This movie relates to Fig. 4A Lower. DLD1 cells overexpressing untagged Mad1 speed through mitosis in, on average, 61.8 min.

Movie S7. This movie relates to Fig. S4. An MDA-MB-231 breast cancer cell expressing histone H2B-RFP (Left) and GFP-tubulin (Center) enters anaphase with a misaligned chromosome, showing that a weakened mitotic checkpoint does occur in a subset of CIN cancer cell lines.

Other Supporting Information Files

SI Appendix (PDF)