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

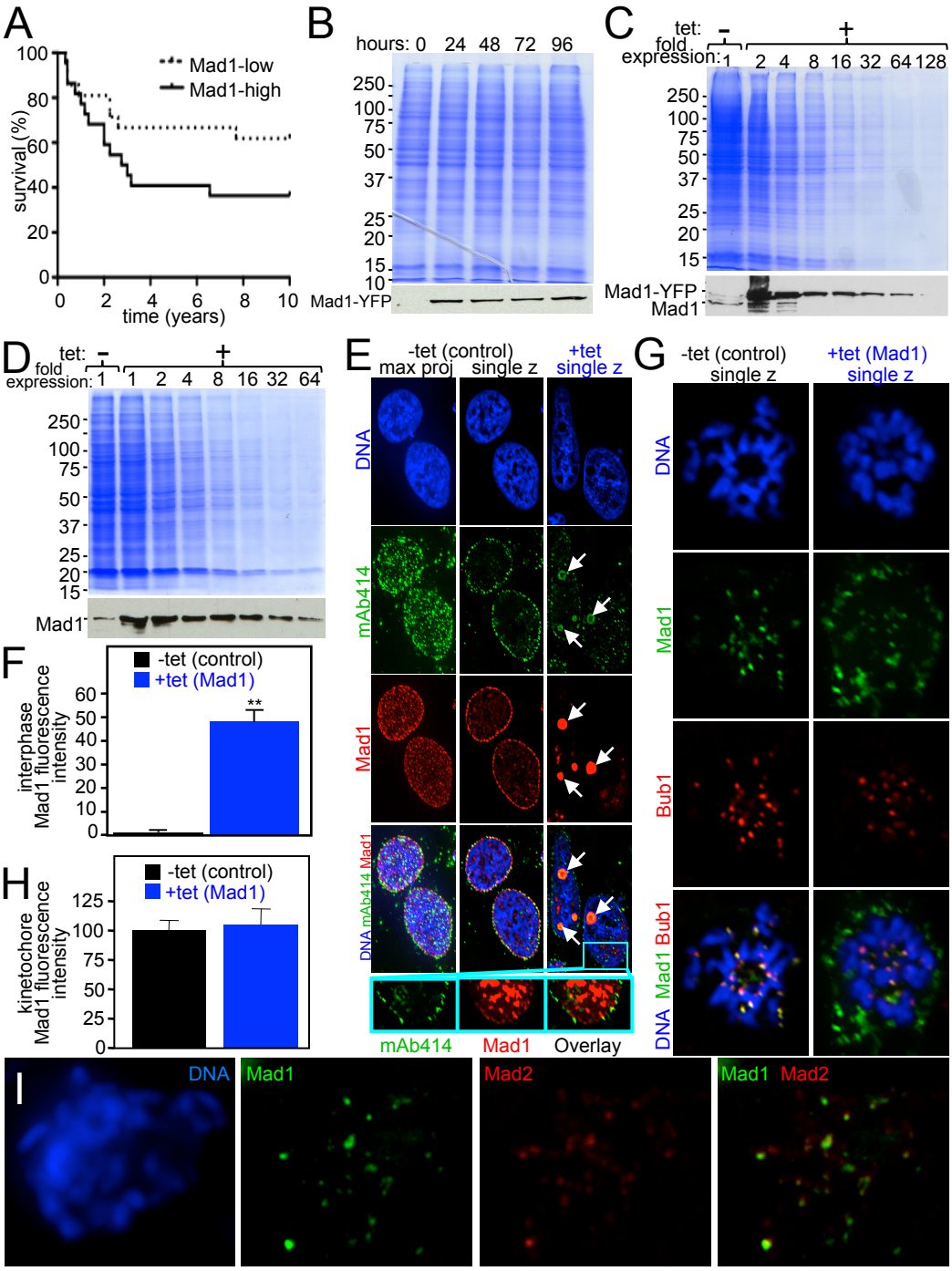

Figure S1. Inducible expression of Mad1 and Mad1-YFP. (A) Colorectal cancer patients with tumors expressing high levels of Mad1 have lower 10 year survival rates than patients with low expression of Mad1. (B) A single administration of tetracycline results in expression of Mad1-YFP protein for ≥4 days. (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. $n \geq 45$ cells from three independent experiments. (I) Single z plane image showing that overexpressed, untagged Mad1 and a portion of Mad2 localize to unattached kinetochores.

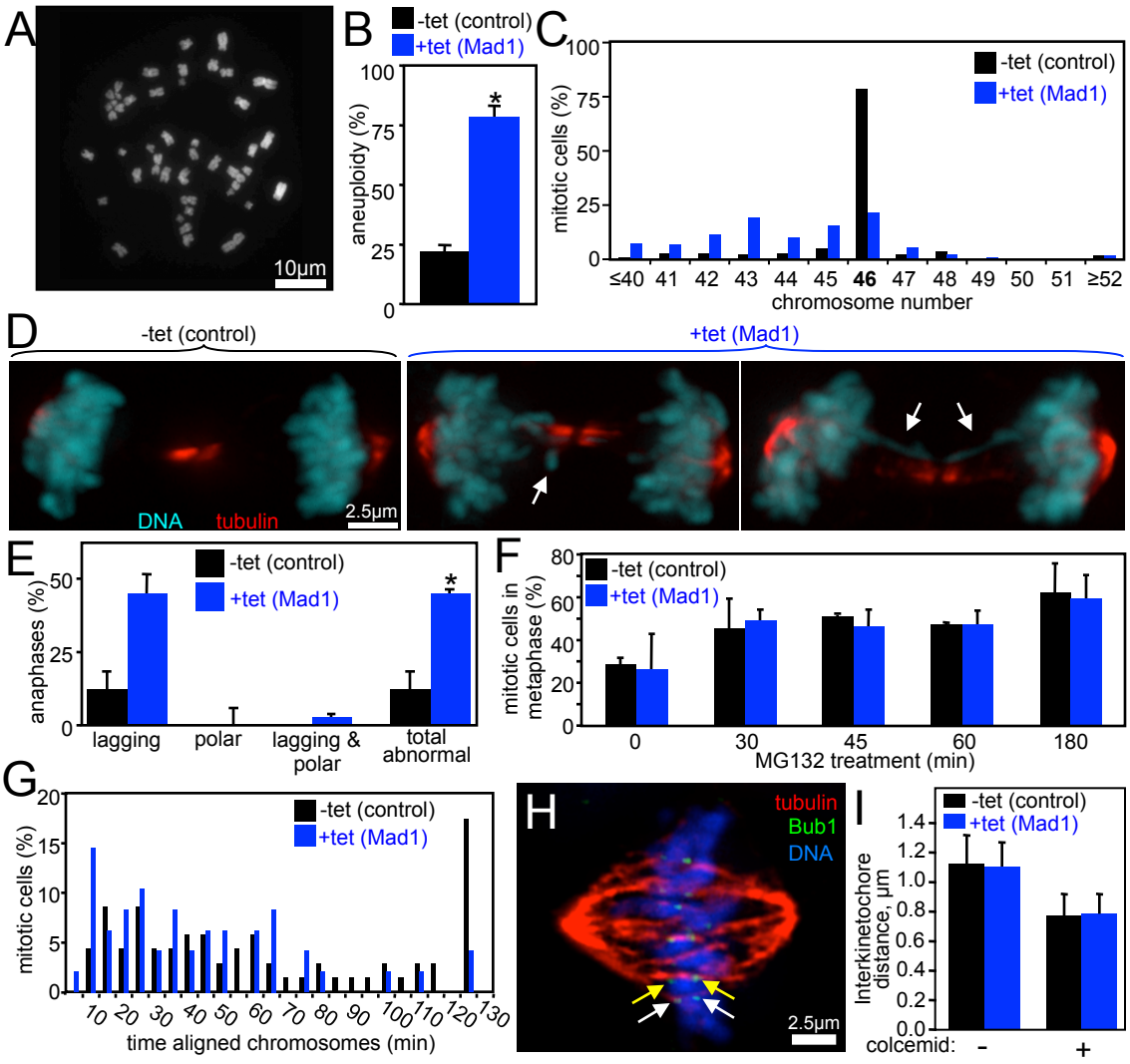

Figure S2. Overexpression of Mad1 results in aneuploidy and chromosomal instability. (A) Chromosome spread of DLD1 cell. Scale bar, 10 μm. (B) DLD1 cells overexpressing Mad1 for 48 hours have higher levels of aneuploidy than control cells. n = 50 cells from each of three independent experiments. * = $p < 0.05$, 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).

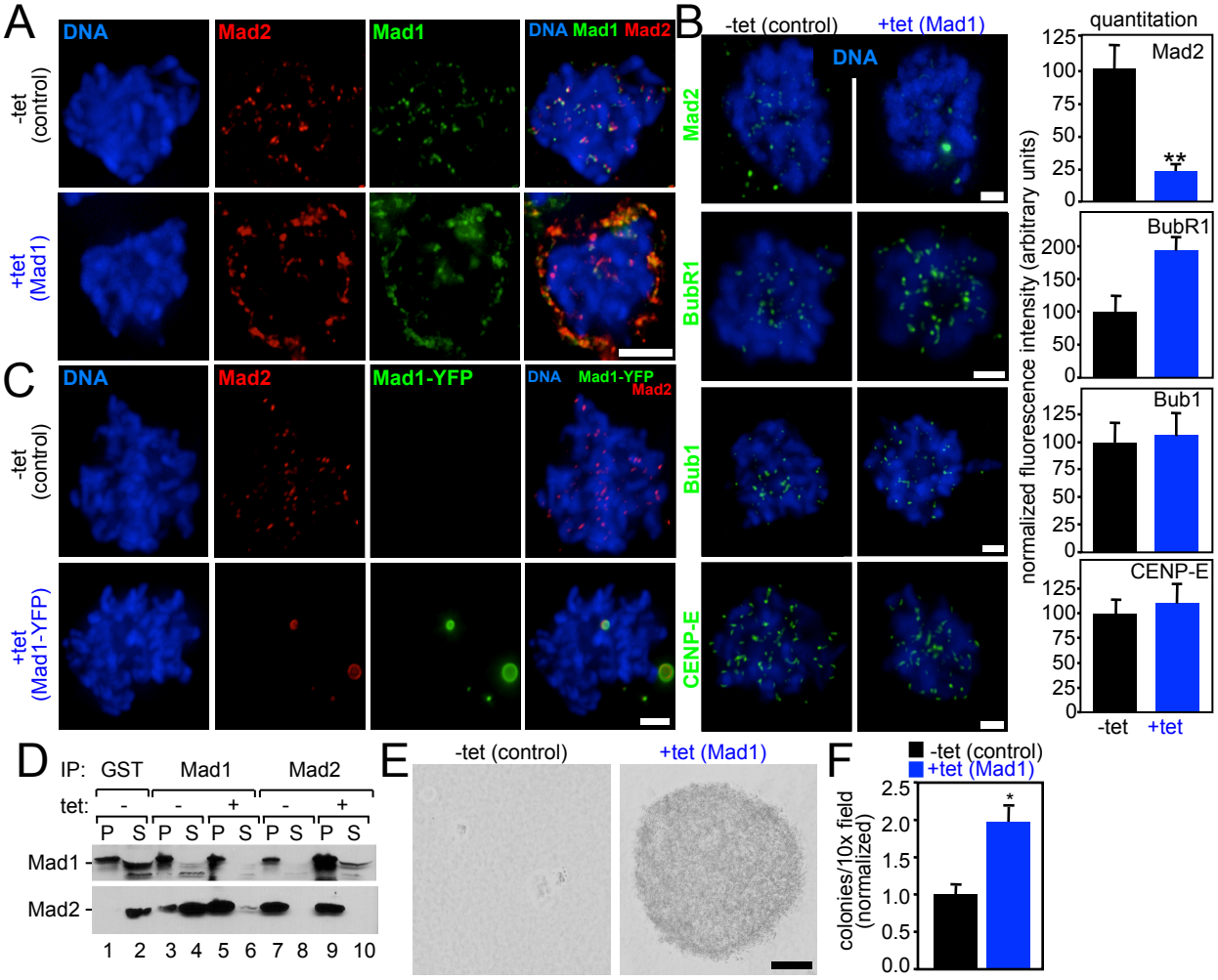

Figure S3. Upregulation of Mad1 mislocalizes Mad2 from kinetochores and enhances transformation. (A) Overexpression of Mad1 (green) largely removes Mad2 (red) from kinetochores. Scale bar, 5 μm. (B) Overexpression of Mad1 mislocalizes Mad2 but not BubR1, Bub1 or CENP-E from kinetochores in an independent 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.

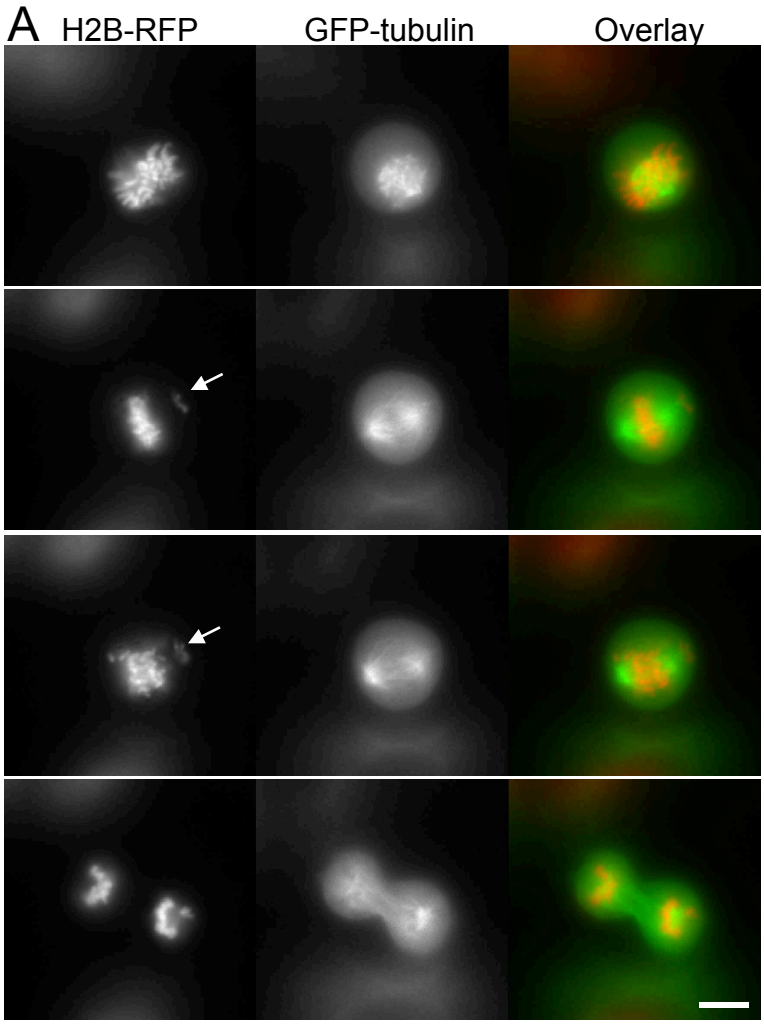

Figure S4. A weakened mitotic checkpoint in human cancer cells. MDA-MB-231 breast cancer cell entering anaphase in the presence of a misaligned chromosome due to a weakened mitotic checkpoint. Still images from timelapse acquisition of histone H2B-RFP (left), GFP-tubulin (center) and the overlay (right) 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.

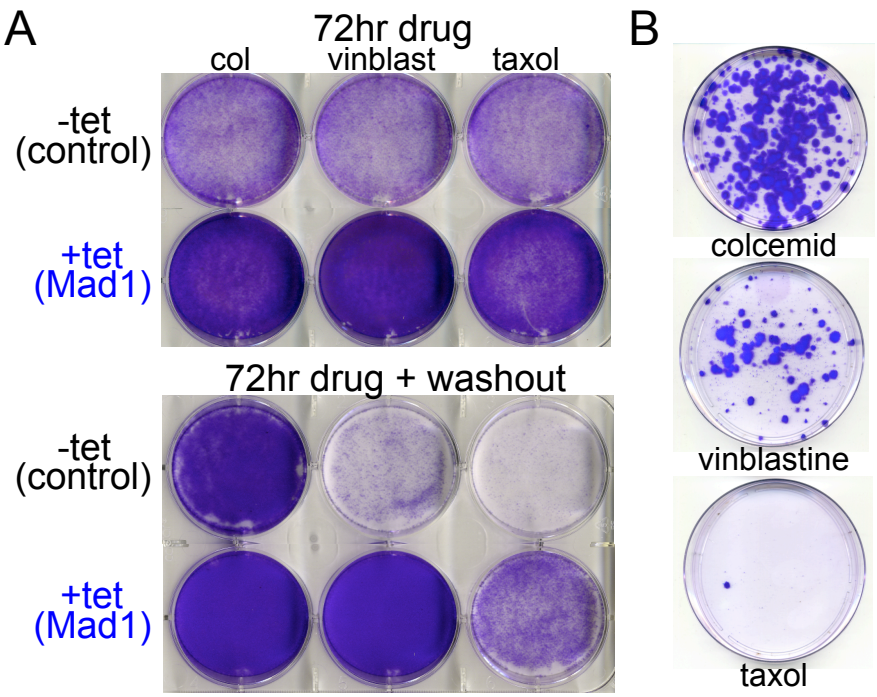

Figure S5. Long-term viability of Mad1-overexpressing cells treated with microtubule poisons. (A) Crystal violet stained dishes of control and Mad1 overexpressing DLD1 cells immediately after 72 hours of treatment with microtubule poisons (top) or after 72 hours of treatment followed by drug washout 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.

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

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 ($p < 0.05$).