Generating chromosome instability through the simultaneous deletion of Mad2 and p53

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Cancer cells exhibit high levels of chromosome instability (CIN), and considerable interest surrounds the possibility that inactivation of the spindle checkpoint is involved. However, homozygous disruption of Mad2 and Bub checkpoint genes in metazoans causes cell death rather than CIN. We now report the isolation and characterization of blastocysts and two independent mouse embryonic fibroblast lines carrying deletions in Mad2 and p53. These cells lack a functional spindle checkpoint, undergo anaphase prematurely, and exhibit an extraordinarily high level of CIN. We conclude that the mitotic checkpoint is not essential for viability per se and that a CIN phenotype can be established in culture through the inactivation of both the Mad2- and p53-dependent checkpoint pathways.

Immunochemistry and Immunoblot Analysis. Cells were fixed, permeabilized, and blocked as described in refs. 24 and 25. Primary incubations with mouse monoclonal Ser-139-phospho-Mad2, Ser-139-phospho-Bub1/Bub3, and Ser-139-phospho-BubR1 antibodies were performed as above.

FACS, Karyotyping, and Live Cell Imaging. For FACS analysis, trypsinized cells were washed in PBS, fixed in 90% ethanol, and stained with propidium iodide (50 μg/ml). Thirty-thousand cells per sample were analyzed by using a FACSCalibur (Becton Dickinson) and standard methods. Polyclonal antiphosphoSer-139 antibodies were used (Molecular Probes), followed by incubation with 1 μg/ml DAPI (Sigma) for 10 min and mounting with Vectashield (Vector Laboratories). Images were acquired as described in ref. 26. Immunoblots were developed by using chemiluminescence (SuperSignal West Femto, Pierce) or a FluoroImager459 (Molecular Dynamics). Images were acquired as described in ref. 26. Immunoblots were developed by using chemiluminescence (SuperSignal West Femto, Pierce) or a FluoroImager459 (Molecular Dynamics) and standard methods. Polyclonal anti-Mad2 peptide immune sera (used at 1:500) were raised against residues 56–50, 117–131, and 194–206.

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Aneuploidy has been recognized as a hallmark of solid cancers since Boveri (1). More recently, it has been shown that cells derived from human colorectal cancers experience frequent chromosome loss and gain, or chromosome instability (CIN) (2). CIN is thought to act as a mutator, increasing the rate at which tumor-promoting mutations are uncovered (3). The molecular basis of CIN remains poorly understood, but considerable interest surrounds the possibility that lesions in spindle checkpoint genes are involved (reviewed in ref. 4). The spindle checkpoint is conserved in higher eukaryotes (reviewed in refs. 5 and 6). An abnormal mitotic entry is nonessential in mammalian cells if p53 also is inactivated, and that simultaneous loss of spindle checkpoint and p53 function creates a CIN phenotype.

Materials and Methods

Analysis of Mice. Mice used in this study were generated from mixed C57BL/6 and SV129 founders, backcrossed five times on C57BL/6, making them at least 80% C57BL/6 (15, 22). Blastocysts were harvested and cultivated as described in ref. 15. DNA was prepared from mouse tails, embryonic yolk sacs, MEFs, or outgrown blastocysts by using standard methods. Genotyping oligonucleotides were used as described in refs. 15 and 22. Animal protocols were approved by the Massachusetts Institute of Technology Committee on Animal Care.

RNA Isolation and RT-PCR. RNA was isolated by using the RNAeasy kit (Qiagen, Valencia, CA). Reverse transcription was performed by using dT18 primers and Superscript II (Invitrogen) according to the manufacturer’s instructions. The following primer sets were used: Mad2-1, (5′-GCACATGGACGCAGAAGCTCAGTCATTGACAGGGG-3′); Mad2-2, (5′-GGCGGCAGAATTCGTCGTC-3′); Mad2-3, (5′-GGCGGCAGAATTCGTCGTC-3′); GAPDH primers as published in ref. 23.

Immunofluorescence and Immunoblot Analysis. Cells were fixed, permeabilized, and blocked as described in refs. 24 and 25. Primary incubations with mouse monoclonal Ser-139-phospho-H2AX antibody (1:400, Upstate Biotechnology, Lake Placid, NY), sheep anti-BubR1, and anti-Bub1 serum (1:1,000, kind gifts from Stephen Taylor, University of Manchester, Manchester, U.K.), or mouse monoclonal anti-α-tubulin (1:500, Tub2.1 Sigma) were performed in PBS/0.2% Triton X-100 with 3% BSA for 1 h at room temperature. Cross-adsorbed, Fluorochrome-labeled secondary antibodies were used (Molecular Probes), followed by incubation with 1 μg/ml DAPI (Sigma) for 10 min and mounting with Vectashield (Vector Laboratories). Images were acquired as described in ref. 26. Immunoblots were developed by using chemiluminescence (SuperSignal West Femto, Pierce) or a FluoroImager459 (Molecular Dynamics) and standard methods. Polyclonal anti-Mad2 peptide immune sera (used at 1:500) were raised against residues 56–50, 117–131, and 194–206.

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Abbreviations: CIN, chromosome instability; MEF, mouse embryonic fibroblast; En, embryonic day; n, inner cell mass; NBiD, nuclear envelope breakdown.

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pressed in embryonic cells by partially or fully inactivating that cell death associated with the loss of blastocysts remained viable for the full 4 weeks of the experiment. Seven of the deletion on the viability of p53 determine whether inactivation of Mad2 was shown. Results

Inactivation of p53 Rescues Mad2−/− Blastocysts from Death. To determine whether inactivation of p53 in mice suppresses lethality associated with Mad2 loss, animals carrying targeted deletions in Mad2 (15) and p53 (22) were intercrossed. None of 241 viable progeny from 50 intercrosses were Mad2-null, regardless of p53 status, demonstrating that Mad2−/− and Mad2−/− p53−/− mice are inviable. To examine the properties of Mad2−/− p53−/− cells, blastocysts were explanted and grown in culture. We have previously shown that cultured Mad2−/− blastocysts die after 5 days, whereas wild-type blastocysts from the same litter grow for many weeks under the same conditions (15). In this study, a total of 102 blastocysts from Mad2 × p53 mutant intercrosses were recovered at E3.5, placed in culture, and examined daily to monitor growth (Fig. 1). All but a subset (see below) were lysed for genotyping after 4 or 5 days in culture (E7.5–E8.5). Consistent with our previous findings, atrophy and death of the proliferating inner cell mass (ICM) was observed in 10 of 10 Mad2−/− p53+/− blastocysts after 3–5 days in culture, even though postmitotic trophodermal cells remained viable (Fig. 1 D and H and data not shown). In contrast, 10 of 11 Mad2−/− blastocysts having either a p53+/+ or p53−/− genotype remained healthy for 5 days in culture and grew at the same rate as Mad2+/− or Mad2+− littermate controls (Fig. 1 A–C and H and data not shown). To examine the long-term effects of p53 deletion on the viability of Mad2-null embryos, 11 littermate blastocysts were grown in culture for a month. Seven of the blastocysts remained viable for the full 4 weeks of the experiment, including one that was Mad2−/−/p53−/− and another that was Mad2−/−/p53+/− (Fig. 1 E–G). We conclude from these data that cell death associated with the loss of Mad2 can be suppressed in embryonic cells by partially or fully inactivating p53. The Generation of Mad2−/− p53−/− MEF Cell Lines. It is difficult to perform detailed cell-cycle studies on explanted embryos, and we, therefore, sought to establish MEF cell lines lacking Mad2 (Fig. 2). MEF lines can routinely be established from wild-type embryos starting at E9.5–E10.5 but not at earlier stages (29). In our original analysis of Mad2−/− mice, no viable embryos older than E7.5 were detected (15). However, by using larger numbers of animals, we now find that up to 15% of Mad2−/− embryos are still viable at E10.5 (Fig. 2 A). Mad2−/− embryos are smaller than littermate controls (data not shown), but their recovery implies that cells can undergo ≥20 divisions in the absence of Mad2, and thus that the establishment of MEFs should be feasible technically. Indeed, harvesting, genotyping, and disaggregation of 208 E10.5 embryos from Mad2−/− p53−/− intercrosses led to the successful establishment of two Mad2−/− MEF lines (Fig. 2 A–C).

The first Mad2-null line arose from a Mad2−/− p53+/− embryo (line AB98; Fig. 2 B and C) and was confirmed to have a Mad2−/− p53+/− genotype after passage in culture; Mad2+/− p53−/− (AB103), and Mad2+/− p53+/− (AB100) littermate control lines were established at the same time. At passage nine, all three lines had similar gross morphologies and doubling times of 20–24 h (Fig. 2D and data not shown), and these characteristics remained true subsequently. No Mad2 mRNA could be detected in AB98 cells by RT-PCR, even after extensive amplification, whereas Mad2 mRNA was readily detectable in AB100 and AB103 cells (Fig. 2E). When protein levels were examined by immunoblotting, Mad2 was found to be abundant in lysates from asynchronous AB100 and NIH 3T3 cells, present at about half the level in AB100 lysates, and undetectable in AB98 lysates (Fig. 2F). Thus, cells carrying homozygous deletions of both Mad2 and p53 and lacking detectable Mad2 mRNA and Mad2 protein are viable in culture. The second Mad2-null line arose from a Mad2−/− p53+/− embryo (line AB152; Fig. 2 B and C), but cells were Mad2−/− p53null when analyzed at passage four (Fig. 2C). AB152 cells grew with a doubling time of nearly 35 h, compared with 20–24 h for NIH 3T3 cells. We surmise that the original Mad2−/− p53+/− embryonic cells had lost their functional copy of p53 upon passage in culture. Thus, a complete loss of functional p53 appears to be necessary for the proliferation of Mad2-null MEFs.

Checkpoint Deficiency in Mad2−/− p53−/− Cell Lines. To establish that Mad2−/− p53−/− MEFs are functionally deficient in the spindle division, including those that were Mad2−/− p53+/− and another that was Mad2−/− p53+/− (Fig. 1 E–G). We conclude from these data that cell death associated with the loss of Mad2 can be suppressed in embryonic cells by partially or fully inactivating p53.
checkpoint, cells were challenged with the microtubule depolymerizing agent nocodazole (Fig. 2G). Wild-type cells respond to nocodazole by arresting in metaphase, whereas cells depleted of Mad2 progress through mitosis rapidly (28, 30–32). The checkpoint status of AB98 (Mad2−/− p53−/−) and AB103 (Mad2+/+ p53−/−) lines was monitored in the presence and absence of nocodazole by using live cell imaging of cells infected with a retrovirus expressing histone H2B–GFP (27) (Fig. 2G). Single cells were monitored for the fraction that had undergone anaphase B at T = 60 min, with nuclear envelope breakdown (NBD) set as T = 0 min (NBD was judged by loss of nuclear integrity and chromosome condensation). One-hundred percent of untreated but only 4% of nocodazole-treated AB103 Mad2+/+ p53−/− cells were observed to have undergone anaphase B by T = 60 min. This reflects the imposition of a cell-cycle arrest. In contrast, 100% of untreated and ~96% of nocodazole-treated AB98 Mad2−/− p53−/− cells had undergone anaphase B at T = 60 min (Fig. 2G). The morphologies of the 4% of Mad2−/− p53−/− cells that failed to segregate their chromosomes by T = 60 min were suggestive of mitotic catastrophe rather than prolonged arrest. Similar data were obtained from AB152 cells (not shown). We conclude from these results that the spindle checkpoint is functionally inactive in Mad2−/− p53−/− cells.

The transient inactivation of Mad2 in HeLa and PK2 cells by antibody injection, expression of dominant negative proteins, or interfering-RNA-mediated depletion not only abrogates the checkpoint but also accelerates the time of anaphase onset sufficiently that a metaphase plate fails to form in more than half of all cells (28, 30, 33, 34). We were, therefore, surprised that mitotic Mad2-null MEFs appeared to contain more or less normal spindles and metaphase plates (Fig. 3 and data not shown). To characterize mitotic progression in these cells, the time of anaphase onset was monitored in at least 30 H2B–GFP-expressing cells from each of two Mad2-null and two Mad2-wildtype cell lines by live cell imaging. We have previously shown that the time of anaphase onset in HeLa cells exhibits a skew-normal distribution whose peak (mode) is the best measure of mitotic progression (28), and the same is true of MEFs. The time of anaphase A onset in AB103 and NIH 3T3 cells varied from 18 to 52 min, with a modal value of 22–23 min (Fig. 3 D and E). In contrast, the peak time of anaphase A onset in AB98 and AB152 Mad2-null cells was 10 min, and no cells waited longer than 20 min (Fig. 3 D and E and data not shown). These values are remarkably similar to the 10-min mode obtained in HeLa cells after Mad2 depletion by using short interfering RNA (28). From these data, we conclude that AB98 and AB152 cells not only lack a functional mitotic checkpoint but also undergo mitosis on a highly accelerated schedule.

Extreme Chromosome Instability in Mad2−/− p53−/− Cells. To determine the effects of checkpoint inactivation on the fidelity of chromosome segregation, anaphase cells from fixed samples of asynchronous cultures were counted and sorted into five different morphological classes: normal, one lagging chromosome, asynchronous cultures were counted and sorted into five different morphological classes: normal, one lagging chromosome, DNA bridges in late anaphase, extreme maloriented chromatid pairs (compared with 18% in Mad2+/+ p53−/−; data not shown). In five cases, lagging chromosomes gave rise to micronuclei in telophase (Fig. 3B and data not shown). In other cells, DNA bridges formed between the separating chro-
mosome masses during anaphase (Fig. 3C and data not shown). Lagging and misaligned chromosomes in AB98 and AB152 cells recruited high levels of Bub1 and BubR1 (Fig. 4C), but the presence of these spindle checkpoint proteins was insufficient to impose a cell-cycle arrest (Fig. 2G), as anticipated from previous interfering RNA data (27, 33). The level of chromosome missegregation in AB100 cells was intermediate between AB98 and AB103 cells (Fig. 4B), presumably reflecting partial inactivation of the checkpoint in Mad2−/− cells (35). AB103 Mad2−/− p53−/− cells also missegregated chromosomes at higher rates than NIH 3T3 or control MEF cells (Fig. 4B), consistent with a requirement for p53 in the G1 checkpoint controlling the division of polyploid cells (36, 37). Importantly, however, the rate of missegregation in Mad2−/− p53−/− cells was much higher than that in Mad2+/+ p53−/− cells, and it appeared that half of all cell divisions in Mad2−/− p53−/− cells were aberrant. Thus, the loss of both p53 and Mad2 in murine cells gives rise to an extreme CIN phenotype.

It has been proposed that polyploidy might provide a genetic buffer allowing cells to better survive the loss of spindle checkpoint function and the consequent increase in CIN (36). To determine whether a connection exists between tetraploidization and survival in the absence of Mad2, we examined the ploidy of our two Mad2−/− MEF lines by FACS and karyotyping (Fig. 4D and E). AB98 cells were found to be largely diploid, although aneuploidy was apparent in the broad distribution of the chromosome number around the euploid value of 40. In contrast, AB152 cells were largely tetraploid and contained 70–90 chromosomes. Thus, both diploid and tetraploid cells can survive in the absence of Mad2 and p53.

Why does p53 deletion suppress the lethality associated with the loss of Mad2? p53 deletion has previously been shown to rescue embryonic lethality resulting from the disruption of genes involved in double-strand break repair, including DNA ligase IV, Xrc4, Rad51, and Brcal (20, 21, 38, 39). By analogy, it seemed possible that abnormal chromosome missegregation in Mad2−/− cells, including the formation of DNA bridges and micronuclei, might generate double-strand DNA breaks that induce p53-mediated apoptosis. To determine whether Mad2−/− p53−/− cells contain high levels of double-strand DNA breaks, we monitored the levels of phospho-H2AX by immunofluorescence and immunoblotting (Fig. 5). Phospho-H2AX is a modified histone that accumulates at double-stranded breaks and acts to recruit DNA repair proteins (40). We observed no significant difference in the

<table>
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<th>Cells</th>
<th>NBD-Anaphase Interval (min)</th>
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<tr>
<td>NIH 3T3</td>
<td>23.1 +/- 1.7</td>
<td>109</td>
</tr>
<tr>
<td>AB103</td>
<td>22.5 +/- 5.1</td>
<td>30</td>
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<tr>
<td>AB103</td>
<td>22.5 +/- 5.1</td>
<td>30</td>
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<tr>
<td>AB98</td>
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<td>44</td>
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<tr>
<td>AB98</td>
<td>10 +/- 0.5</td>
<td>53</td>
</tr>
<tr>
<td>AB152</td>
<td>10 +/- 0.5</td>
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Fig. 3. Live cell imaging and accelerated mitotic progression in cells lacking Mad2. (A–C) Images of Mad2+/+ p53−/− (AB103) and Mad2−/− p53−/− (AB98) during mitosis by using time-lapse microscopy. Chromatin was visualized with H2B–GFP. The T = 0 min time point shows cells shortly before NBD, as judged by loss of nuclear integrity and compact organization of the chromosomes. Cells were imaged every 2 min from this point until chromosome decondensation after anaphase. Pictures were taken every 2 min using a ×63 objective on a DeltaVision microscope (Zeiss). (Scale bars: 10 μm.) M indicates a lagging chromosome that seems to form a micronucleus in anaphase. (D) Quantitation of data from live cell imaging. Images were taken every 4 min with a ×20 objective. The time between NBD and anaphase onset was determined for at least 30 cells and is shown as accumulative graph. Mad2−/− p53−/− cell lines AB98 and AB152 (shown as the average of both cell lines) show accelerated progression although mitosis, compared with Mad2+/+ p53−/− (AB103) and NIH 3T3 cells. (E) Summary of mitotic timing data presented in D. In each case, the mode of the time of anaphase onset is shown. The error in mitotic times was estimated by subsampling the data and determining the standard deviation in the mode; n shows the total number of cells that were analyzed.
levels of phospho-H2AX in normally growing AB98 (Mad2<sup>−/−</sup> p53<sup>−/−</sup>) and AB103 (Mad2<sup>−/−</sup> p53<sup>−/−</sup>) cells. However, both lines exhibited a ≥10-fold induction of phospho-H2AX after irradiation (Fig. 5). For reasons that are not clear, the induced levels of phospho-H2AX in AB98 and AB100 (Mad2<sup>−/−</sup> p53<sup>−/−</sup>) cells were 30–40% lower than that in NIH 3T3 and AB103 control cells (Fig. 5B). Nonetheless, we conclude that deletion of Mad2 does not lead to the accumulation of high levels of DNA double-strand breaks, at least as assayed by phospho-H2AX accumulation.

**Discussion**

Mouse knockout studies have shown that Mad2, BubR1, and Bub3 are essential for the viability of mice and of murine cells (15–17). The essential nature of spindle checkpoint genes is widely assumed to reflect a requirement for the checkpoint in the orderly execution of the mechanical events of mitosis. Consistent with this assumption, transient inactivation of Mad2 by using antibody microinjection, interfering RNA, and dominant negative protein fragments causes such a dramatic acceleration of mitosis that chromosomes fail to make stable bipolar attachments to microtubules and the metaphase plate fails to form (28, 30, 33, 41). However, we show in this paper that lethality associated with deletion of Mad2 can be suppressed by the additional deletion of p53 to yield viable Mad2<sup>−/−</sup> p53<sup>−/−</sup> cells. The inability of Mad2<sup>−/−</sup> p53<sup>−/−</sup> MEFs to arrest in response to the microtubule poison nocodazole and the decoupling of anaphase onset from the completion of chromosome-microtubule attachment demonstrate that the spindle checkpoint is functionally inactive in these cells. Mad2<sup>−/−</sup> p53<sup>−/−</sup> MEFs grow in culture with a doubling time similar to that of control p53<sup>−/−</sup> MEFs, even though they experience high levels of chromosome missegregation. Thus, Mad2<sup>−/−</sup> cells appear to die primarily as a consequence of apoptosis (regulated at least in part by p53) rather than because of mitotic catastrophe per se.

Our findings help to resolve a longstanding debate as to why the spindle checkpoint is essential for the survival of mammalian cells but not for yeast (7, 8, 13–17). Two major differences between multi- and unicellular eukaryotes are probably involved. First, p53-dependent apoptosis does not exist in yeast (42, 43), and our results argue that apoptosis observed in Mad2<sup>−/−</sup> embryos requires p53. Second, yeast chromosomes are attached to microtubules throughout the cell cycle, whereas chromosomes in higher cells bind to microtubules only during mitosis (reviewed in ref. 44). In yeast, Mad2p appears to be recruited to kinetochores only on the rare occasion that a chromosome becomes detached from microtubules (45). In contrast, Mad2 is present on prometaphase mammalian-cell kinetochores during every cell division (32, 46) and regulates mitotic progression even in the absence of exogenous spindle damage (28).

**Interactions Between p53 and Mad2.** The extent to which p53 deletion suppresses death associated with Mad2 loss varies with the biological setting. In blastocysts grown in vitro, Mad2<sup>−/−</sup>-embryonic cells are viable if they carry either a heterozygous or homozygous deletion of p53. In embryonic fibroblasts, it appears that very strong selective pressure is exerted on Mad2<sup>−/−</sup> cells to lose both copies of p53: only 1 in 10 Mad2<sup>−/−</sup> p53<sup>−/−</sup> embryos examined yielded a cell line, and these cells had apparently undergone loss of heterozygosity to become p53-null. Finally, even homozygous p53 deletion does not result in the birth of Mad2-null mice. It might seem surprising that a p53 heterozygous state is sufficient to suppress cell death in Mad2<sup>−/−</sup> embryos, but...
similar effects have been reported in cells lacking DNA ligase and XRCC4, suggesting that cells are sensitive to even small changes in the levels of p53 (20, 21).

The existence of two cell lines that lack a Mad2 gene, protein, and mRNA establishes that murine cells can survive in the number of lines raises the question of whether p53 loss is really necessary and sufficient for the survival of checkpoint deficient cells. The most convincing data that p53 loss is necessary comes from an analysis of blastocysts: 0 of 10 Mad2−/− p53+/+ blastocysts cultured in vitro were alive at E8.5, compared with 10 of 11 Mad2−/− p53−/− and Mad2−/− p53−/− blastocysts. Thus, p53 deletion clearly has a significant effect on the viability of Mad2-null cells, although it remains to be established whether the deletion of genes upstream and downstream of p53 has a similar effect. Our data do not clearly establish whether p53 loss is sufficient for the survival of Mad2-deficient cells. Because checkpoint-deficient cells exhibit extremely high levels of chromosome missegregation, they are certain to accumulate many mutations. We cannot rule out the possibility that one or more genetic alterations in addition to p53 loss are required for the survival of cells lacking a spindle checkpoint.

When Mad2 is deleted in the death of cells lacking Mad2? The loss of p53 might facilitate the establishment of Mad2−/− cell lines simply by promoting immortalization, but we also observe an effect of p53 loss in blastocysts in which immortalization is not an issue. Alternatively, p53 deletion might allow Mad2−/− cells to bypass a G2 checkpoint proposed to operate in cells that exit abnormally from mitosis (but see ref. 47 for another view). Although a negative result, our inability to detect elevated levels of phospho-H2AX in Mad2−/− p53−/− cells implies that DNA damage may not be the primary apoptotic trigger in checkpoint-deficient cells. It is, therefore, appealing to speculate that the proposed role of p53 in sensing ploidy per se might be involved; further analysis of p53 regulators such as ATM/ATR should help to resolve this issue (48).

Generating a CIN Phenotype. A large fraction of human solid tumors are aneuploid (2, 49), and it is reasonable to propose that CIN is a primary cause of this aneuploidy. The mutations that cause CIN are unknown, but a high percentage of tumor cells fail to arrest in response to microtubule poisons such as nocodazole (reviewed in ref. 50). This finding has prompted a search for mutations in spindle checkpoint genes associated with tumorigenesis. Such mutations have been found in some human CIN lines (51), but it has been disappointing that loss-of-function mutations engineered into mouse Mad and Bub genes cause cell death rather than CIN. The data in this paper suggest that generating a CIN phenotype requires knocking out two checkpoint pathways: one involving p53 and the other involving the Mad2-dependent pathway. Cells lacking Mad2 and p53 change chromosome numbers so rapidly that we have not managed to measure rates of missegregation precisely. Imaging and karyotyping suggest one or more chromosome loss-and-gain events per cell division. Taken at face value, this finding appears to establish a strong connection between CIN and checkpoint loss, at least in cells grown in culture.

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