BUB1 mediation of caspase-independent mitotic death determines cell fate

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The spindle checkpoint that monitors kinetochore–microtubule attachment has been implicated in tumorigenesis; however, the relation between the spindle checkpoint and cell death remains obscure. In BUB1-deficient (but not MAD2-deficient) cells, conditions that activate the spindle checkpoint (i.e., cold shock or treatment with nocodazole, paclitaxel, or 17-AAG) induced DNA fragmentation during early mitosis. This mitotic cell death was independent of caspase activation; therefore, we named it caspase-independent mitotic death (CIMD). CIMD depends on p73, a homologue of p53, but not on p53. CIMD also depends on apoptosis-inducing factor and endonuclease G, which are effectors of caspase-independent cell death. Treatment with nocodazole, paclitaxel, or 17-AAG induced CIMD in cell lines derived from colon tumors with chromosome instability, but not in cells from colon tumors with microsatellite instability. This result was due to low BUB1 expression in the former cell lines. When BUB1 is completely depleted, aneuploidy rather than CIMD occurs. These results suggest that cells prone to substantial chromosome missegregation might be eliminated via CIMD.

Introduction

Defects in the attachment of microtubules to kinetochores activate the spindle checkpoint to delay mitotic progression by transiently inhibiting the anaphase-promoting complex (also called the cyclosome) (Rieder and Maiato, 2004). Genes involved in the spindle checkpoint were first isolated from Saccharomyces cerevisiae and include MAD1, MAD2, and MAD3 (mitotic arrest–deficient) (Li and Murray, 1991); BUB1, BUB2, and BUB3 (budding uninhibited by benzimidazole [a microtubule-depolymerizing drug]) (Hoyt et al., 1991); and MPS1 (monopolar spindle) (Wells and Murray, 1996).

The spindle checkpoint proteins and their functions are highly conserved between yeast and humans, and defects in the spindle checkpoint result in substantial aneuploidy (Kitagawa and Hieter, 2001; Kops et al., 2005). Much evidence also indicates a role of the spindle checkpoint in tumorigenesis, e.g., mutations in human homologues of Bub1 (BUB1 and BUBR1) have been found in subtypes of colorectal cancer cells that exhibit chromosome instability (CIN) (Cahil et al., 1998). The CIN phenotype has been associated with mutations in spindle checkpoint genes (Ohshima et al., 2000; Tsukasaki et al., 2001; Ru et al., 2002), decreased levels of spindle checkpoint proteins (Shigeishi et al., 2001; Saeki et al., 2002), and loss of spindle checkpoint activity (Wang et al., 2002; Yoon et al., 2002). Mad2−/− mice frequently develop lung tumors after a long latency (Michel et al., 2001). Bubrs−/− mice and Bub3/Rae1 heterozygotes are prone to tumor development (Babu et al., 2003; Dai et al., 2004). These results strongly suggest a close relation between altered activity of the spindle checkpoint and tumorigenesis. Also, many tumor cells have a diminished, but not absent, spindle checkpoint response (Kops et al., 2005). When the function of mouse Bub1 is compromised cells appear to escape apoptosis and continue to progress through the cell cycle, despite leaving mitosis with an altered spindle (Taylor and McKeon, 1997). However, opposing evidence indicates that the spindle checkpoint regulates apoptosis: mutations in bub1 cause chromosome missegregation and fail to block apoptosis in Drosophila (Basu et al., 1999), and Mad2-null mouse embryos undergo apoptosis at embryonic day (E) 6.5 to E7.5 (Dobles et al., 2000). In all of these cases, apoptosis appeared to occur in the subsequent G1 phase; thus, the role of the spindle checkpoint in apoptosis remained unclear.

When cells cannot satisfy the spindle checkpoint after a long mitotic delay, several cell fates can occur: some cells die during mitosis, some exit mitosis but die via apoptosis in the detectability of caspase-independent mitotic death (CIMD).
G1 phase, and some exit mitosis but are tetraploid and reproductively dead (Rieder and Maiato, 2004). Microtubule inhibitors induce mitotic arrest by activating the spindle checkpoint; eventually, these inhibitors cause cytotoxicity. The cytotoxicity of microtubule inhibitors and resultant cell death has been described as either apoptosis in G1 or reproductive death (Mollinedo and Gajate, 2003). However, questions about cell death during mitosis have remained. Although much evidence suggests that apoptosis occurs during mitosis (Woods et al., 1995; DeLuca et al., 2002; Burns et al., 2003; Yang et al., 2005; Blank et al., 2006), in-depth analyses of mitotic cell death have not been performed; therefore, the mechanism involved remains obscure, especially the relation between the spindle checkpoint and cell death during mitosis. Here, we report the mechanism of the programmed cell death in early mitosis that is induced by defects in the kinetochore–microtubule attachment in BUB1-deficient cells.

Results

Substantial BUB1 depletion does not affect mitotic delay induced by defects in kinetochore-microtubule attachment

Treatment with nocodazole (a microtubule-depolymerizing drug), paclitaxel (Taxol, a microtubule-stabilizing drug) (Mollinedo and Gajate, 2003), or 17-allylamino-deranamycin (17-AAG, an HSP90 inhibitor that induces delocalization of several kinetochore proteins from kinetochores) (Niikura et al., 2006) caused substantial mitotic delay (Fig. 1 A). We depleted HeLa cells of either BUB1 or MAD2 (both are spindle checkpoint components) by treating the cells with synthetic small interfering RNA (siRNA) (Fig. 1 B). The cells were then incubated in nocodazole, paclitaxel, or 17-AAG to induce mitotic arrest. The depletion of MAD2 (but not BUB1) substantially diminished the arrest (Fig. 1 A). These results are consistent with the finding by Johnson et al. (2004), i.e., depletion of BUB1 does not compromise the mitotic delay either during normal mitosis or in response to spindle damage induced by nocodazole (Johnson et al., 2004). Although a small remaining quantity of BUB1 may be sufficient to induce mitotic delay (Meraldi and Sorger, 2005), ~90% depletion of BUB1 did not affect mitotic delay induced by defects in kinetochore–microtubule attachment.

Depletion of BUB1 or MAD2 sensitizes cells to 17-AAG or paclitaxel

Synthetic lethality occurs between spindle checkpoint mutants and kinetochore mutants in yeast, presumably because of synergistic, substantial chromosome loss (Hyland et al., 1999; Tong et al., 2001). In human cells, simultaneous depletion of the kinetochore protein HEC1 and MAD2 causes premature catastrophic exit from mitosis (Martin-Lluesma et al., 2002). Because 17-AAG causes kinetochore defects (Niikura et al., 2006), we examined whether 17-AAG treatment in conjunction with defects in the spindle checkpoint induces synthetic lethality. MAD2 or BUB1 was depleted from HeLa cells by siRNA treatment. The cells were then exposed to 100 nM 17-AAG, which did not kill most control cells, and viability was evaluated by a colony outgrowth assay (Fig. 1 C, top). The proportion of cells killed by MAD2 siRNA or BUB1 siRNA alone did not differ from that seen with control luciferase siRNA, but treatment with either MAD2 siRNA or BUB1 siRNA and 17-AAG caused substantial synergistic lethality (Fig. 1 C).
Spindle checkpoint mutants in yeast are also sensitive to microtubule inhibitors (Hoyt et al., 1991; Li and Murray, 1991), presumably because of synergistic substantial chromosome loss. Therefore, we examined whether paclitaxel treatment, in conjunction with defects in the spindle checkpoint, induces synthetic lethality. Like 17-AAG, paclitaxel with either MAD2 siRNA or BUB1 siRNA caused substantial synergistic lethality (Fig. 1 C). Death induced by MAD2 siRNA and 17-AAG or paclitaxel is presumably caused by the failure of checkpoint-induced mitotic arrest, which results in premature mitotic exit and synergistic aneuploidy (Fig. 2 A). The resulting abnormal nuclei (i.e., fragmented/aggregated nuclei, micronuclei, or chromosome bridges; Fig. 2 B) are similar to those of cells that are MAD2 depleted for several cell divisions (Kops et al., 2004); this lethal phenotype can be explained by premature mitotic exit, i.e., the current understanding of how the spindle checkpoint protects cells from aneuploidy (Mollinedo and Gajate, 2003) (Fig. 2 A). Moreover, the abnormal nuclear phenotypes are associated with the degree of MAD2 depletion (Fig. 2, C and D). However, BUB1-depleted cells treated with 17-AAG or paclitaxel did not appear to exit mitosis (Fig. 1 A). This finding raises a provoking question: How does simultaneous treatment with BUB1 siRNA and 17-AAG or paclitaxel cause substantial synergistic lethality, when BUB1 depletion does not cause premature mitotic exit? The spindle checkpoint appeared functional (Fig. 3 A).

**Microtubule inhibitors or 17-AAG induce mitotic cell death in BUB1-depleted cells**

To evaluate the lethal phenotype caused by 17-AAG and either MAD2 siRNA or BUB1 siRNA, we used the TUNEL assay. When cells were treated simultaneously with 17-AAG and BUB1 siRNA, but not with MAD2 siRNA, most of the TUNEL+ cells were in prophase, prometaphase, or metaphase (Fig. 3 E). To exclude the possibility of off-targets of siRNA, we used several siRNA oligos to induce DNA fragmentation (unpublished data; see Fig. S4 A, available at http://www.jcb.org/cgi/content/full/jcb.200702134/DC1). Overexpression of BUB1 suppressed DNA fragmentation when siRNA targeted the 3′ UTR region of BUB1 (Fig. S1, A and B). Although the BUB1-depleted cells appeared to be arrested in mitosis, they must have been dead or dying, because the DNA had already fragmented...
Figure 3. CIMD occurs in BUB1-depleted cells in the presence of microtubule inhibitors or 17-AAG. (A) A model showing BUB1-depleted cells. When cells had defective kinetochore–microtubule attachment, mitotic delay occurred, and the spindle checkpoint appeared to be active (ON). The substantial synthetic lethality cannot be explained because there is no premature exit from mitosis. (B) HeLa cells that are BUB1-depleted and 17-AAG–treated exhibit DNA fragmentation (TUNEL) during mitosis. 48 h after HeLa cells were transfected with siRNA against MAD2, BUB1, or Luc, they were incubated with 17-AAG (+17-AAG, 500 nM) for 24 h at 37°C. Fixed samples were stained by using an in situ cell death detection system that contained TMR red (red), an anti-phosphorylated histone H3 (p-H3) mouse monoclonal antibody, and FITC-conjugated secondary antibodies (green). DNA was stained with DAPI (blue) to visualize prophase, prometaphase, and metaphase cells. Bar, 10 μm. (C) A histogram summarizing TUNEL assay results of BUB1- or MAD2-depleted cells. HeLa cells transfected with siRNA against MAD2, BUB1, or Luc were treated with 17-AAG (500 nM), NOC (0.5 μg/ml), or Taxol (10 nM) for 24 h at 37°C. DNA fragmentation was detected by the TUNEL assay, and samples underwent indirect fluorescence microscopy using anti-p-H3 as a primary antibody. More than 200 cells in three independent experiments were counted, and the mean percentages (± SD) of TUNEL+ cells and mitotic TUNEL cells (mitotic cells were those that were positive for p-H3 and had characteristic chromosome morphology) were calculated. Gray bars represent the mean percentages of TUNEL+ cells in the population, and black bars indicate the mean percentages of mitotic TUNEL+ cells. (D) Almost 90% of the BUB1-depleted mitotic cells that were treated with 17-AAG, NOC, or Taxol were TUNEL+. A histogram summarizing TUNEL assay results of 17-AAG–treated and BUB1- or MAD2-depleted mitotic cells is shown in C. The number of TUNEL+ cells among more than 200 mitotic cells was counted, and the percentages (i.e., the number of mitotic TUNEL+ cells per that of total mitotic cells) are shown. MAD2 or Luc siRNA did not induce any mitotic TUNEL+ cells. (E) DNA fragmentation in BUB1-depleted and 17-AAG–treated mitotic HeLa cells was detected by electrophoresis. 48 h after HeLa cells were transfected with BUB1 siRNA, they were treated with 17-AAG (500 nM) for 6 h. Mitotic cells were isolated by pipetting (~90% of the isolated population consisted of mitotic cells), and DNA was extracted and subjected to electrophoresis in a 1% agarose gel (lane 2). As a negative control, DNA extracted from mitotic HeLa cells treated with 17-AAG (500 nM) was loaded (lane 1); and as a positive control, we loaded DNA extracted from HeLa cells treated with staurosporine (1 μM), a known inducer of apoptosis (lane 3). The molecular size markers (1-kb DNA ladder; New England Biolabs) are indicated (lane M). Fragmented DNA prepared from the same amount of cells was loaded into each lane. Our method of DNA isolation isolated only fragmented DNA; therefore, if cells contained little or no fragmented DNA, the same was observed in that lane. (F) A model showing BUB1-depleted cells in which defects in kinetochore–microtubule attachment induce lethal DNA fragmentation. Because cells are still arrested in mitosis, the mitotic index is unchanged. Therefore, the spindle checkpoint appears to be active (ON).

During early mitosis (Fig. 3 F). This finding answers the question posed above.

Cells treated with BUB1 siRNA and either nocodazole or paclitaxel underwent mitotic cell death (Fig. 3 C; Fig. S1 C). Interestingly, ~90% of the mitotic cells were TUNEL+ (Fig. 3 D). These drugs commonly cause defective kinetochore–microtubule attachment. Therefore, these results strongly suggest that this mitotic cell death occurs when the kinetochore–microtubule attachment is altered and BUB1 function is disrupted.

Caspase-independent mitotic death

We detected no caspase activity (caspases 1, 3–9) in cells exposed to 17-AAG and BUB1 siRNA (Fig. 4 A). Furthermore, caspase inhibitors BAF and zVAD did not inhibit DNA fragmentation induced by 17-AAG and BUB1 siRNA (Fig. 4 B, see Fig. S1 D for drug evaluation controls). Therefore, this mitotic cell death was caspase independent. Apoptosis caused by spindle checkpoint defects is thought to occur during the G1 phase, and the type of cell death that we identified does not meet the criteria for other defined types of cell death (Okada and Mak, 2004); thus, we designated this type of cell death as caspase-independent mitotic death (CIMD).

Because CIMD occurs in HeLa cells with compromised p53 activity (Hoppe-Seyler and Butz, 1993), CIMD appeared to be independent of p53. We confirmed that CIMD occurs in cells that lack p53 (Figs. 4 C; Fig. S1 E and Table S1). Next, we examined whether CIMD depends on p73, a homologue of p53, because a mitotic function of p73 has been suggested (Fulco et al., 2003; Merlo et al., 2005). Overexpression of the dominant-negative mutant p73DD (Irwin et al., 2000) suppressed CIMD.
Mitochondria release apoptosis-inducing factor (AIF) and endonuclease G (EndoG) (Susin et al., 1999; Li and Hoffman, 2001; van Loo et al., 2002), which are thought to regulate caspase-independent cell death (Susin et al., 2000; Joza et al., 2001; Cregan et al., 2002; Yu et al., 2002). Therefore, we examined whether AIF and EndoG are required for CIMD. Substantial amounts of AIF and EndoG were released from mitochondria in mitotic cells treated with 17-AAG and BUB1 siRNA (Fig. 5, A and C). AIF and EndoG immunostaining resulted in a pattern that resembled that of mitochondria stained with 3,3′-dihexyloxycarbocyanine iodide (DiOC6) in mitotic cells, as described previously (Barni et al., 1996). We confirmed that AIF and EndoG immunostaining was colocalized with MitoTracker Red CM-HsXRos staining (Fig. S2 B). The proportion of AIF- and EndoG-releasing mitotic cells was comparable to that of cells undergoing CIMD (compare Fig. 5, B and D with Fig. 4 B); this similarity strongly suggests that AIF and EndoG are effectors of CIMD.

Next, we examined whether CIMD depends on AIF and EndoG. Depletion of AIF and EndoG by siRNA treatment substantially reduced TUNEL signals that were induced by 17-AAG treatment and BUB1 depletion (Fig. 5 E; Fig. S2 C), whereas depletion of AIF, EndoG, or both did not affect the mitotic delay induced by 17-AAG (Fig. S2 D). These results indicate that DNA fragmentation is dependent on AIF and EndoG.

We examined whether depletion of EndoG and AIF rescues the lethality caused by CIMD. Although depletion of AIF or EndoG alone did not rescue the lethality, of both EndoG and AIF substantially suppressed it (Fig. 5 F), indicating that both effectors are involved in the death-signaling pathway of CIMD. These findings lead us to conclude that CIMD is an active cell death system mediated by these apoptosis effectors.

**CIMD occurs rapidly after the kinetochore-microtubule attachment is altered**

Inhibition of DNA decatenation arrests cells at metaphase, and the disruption of MAD2, but not BUB1, suppresses this metaphase arrest (Skoufias et al., 2004). Therefore, we examined whether CIMD occurs when BUB1-depleted cells are arrested with ICRF187, a topoisomerase II inhibitor. Although we observed a substantial mitotic delay after ICRF187 treatment (unpublished data), the number of TUNEL+ BUB1-depleted cells was unchanged (Fig. S2 E). This finding suggests that inhibition of DNA decatenation does not induce CIMD and supports the hypothesis that CIMD occurs specifically when the kinetochore-microtubule attachment is altered.

To investigate the timing of CIMD after the kinetochore-microtubule attachment is altered, we added 17-AAG or microtubule inhibitors to BUB1-depleted cells that were arrested by ICRF187. We then monitored TUNEL+ cells. CIMD began to occur within 20 min, and most of the mitotic cells were TUNEL+ within 2 h (Fig. 6 A). This finding indicates that CIMD occurs during mitosis and relatively rapidly after the kinetochore-microtubule attachment is altered, which supports our conclusion that CIMD is an active cell death system.

We also tested whether cold shock induces CIMD. Cold treatment depolymerizes microtubules, which activates the spindle checkpoint (Rieder and Cole, 2002). When cells were incubated at 23°C, CIMD occurred in >90% of the mitotic cells within 3 h (Fig. 6 B). This is the fourth piece of evidence that supports the hypothesis that CIMD is caused by defects in kinetochore-microtubule attachment when BUB1 function is disrupted.
The fate of cells in which CIMD occurred

To learn the fate of cells in which DNA was fragmented during mitosis, we performed time-lapse experiments. Most BUB1-depleted cells that remained in mitosis for 6 h after the addition of 17-AAG eventually collapsed directly from mitosis within 12 h (Fig. 6, C–E). In contrast, most luciferase siRNA–treated cells (a negative control) remained in mitosis up to 12 h later (Fig. 6, C and D). Therefore, the cells in which CIMD occurred looked...
normally arrested in mitosis for several hours after the kinetochore–microtubule attachment was altered.

Most conventional apoptosis detection methods (i.e., annexin V assay, chromatin condensation, and other morphologic analyses by light microscopy) were not applicable to mitotic cells (unpublished data). Therefore, we performed transmission electron microscopy (TEM) to look at the ultrastructural features of cells in which CIMD had occurred. When DNA fragmentation was induced by BUB1 depletion and 17-AAG or paclitaxel, we observed increased numbers of abnormal mitochondria (condensed, whorled, or onion-skin) and autophagosomes (Fig. 6 F; Fig. S3, A and C, available at http://www.jcb.org/cgi/content/full/jcb.200702134/DC1). The mitochondria were significantly smaller than those in control cells (Fig. 6 G), suggesting that mitochondrial fragmentation occurred. These changes indicated active cell death, possibly through autophagy (Perkins et al., 2004; Eskelinen, 2005; Barsoum et al., 2006; Perez et al., 2007).

Partial, but not complete, depletion of BUB1 causes CIMD

BUB1 depletion does not compromise mitotic delay during normal mitosis or in response to nocodazole-induced spindle damage (Johnson et al., 2004). Our findings support that earlier study, and we believe that we can now explain this phenomenon. Because CIMD occurred, the mitotic index appeared to be unchanged. When a small amount of BUB1 remains in the cell
it is sufficient to induce mitotic delay, but when BUB1 is completely depleted, cells prematurely exit mitosis (Meraldi and Sorger, 2005). Therefore, we attempted to determine how much BUB1 would have to be depleted to induce CIMD.

We performed an siRNA dilution experiment using BUB1 targets to deplete BUB1 almost completely. When BUB1 was nearly depleted, CIMD occurred or the mitotic index was significantly reduced (Fig. 7, A and B; Fig. S4 A, available at http://www.jcb.org/cgi/content/full/jcb.200702134/DC1). Therefore, complete depletion of BUB1 causes premature mitotic exit. The number of abnormal nuclei was also increased similarly to that seen after MAD2 depletion (Fig. 7 C), and partial depletion of MAD2 did not induce CIMD (Fig. 2; Fig. S4 B).

These results indicate that CIMD does not occur when BUB1 is almost completely depleted; the remaining BUB1 appears to be required to induce CIMD. A substantial number of cells with abnormal nuclei did not result from CIMD, which raises the possibility that CIMD might kill the cells that are going to have abnormal nuclei. Furthermore, a kinase-dead BUB1 mutant failed to suppress CIMD, which suggests that the kinase activity is important for inhibition of CIMD (Fig. 7 D).

CIMD is a major cell death mechanism of tumors with CIN that is induced by microtubule inhibitors or 17-AAG

CIMD depends on BUB1 depletion, which suggests that microtubule inhibitors or 17-AAG induces CIMD of tumor cells that have a deficient spindle checkpoint. We tested whether microtubule inhibitors or 17-AAG induces CIMD of cells derived from tumors with CIN in which the spindle checkpoint is compromised and of tumor cells with microsatellite instability (MIN) in which the spindle checkpoint is intact (Cahill et al., 1998).

CIMD occurred in tumor cell lines with CIN (Caco-2, SW480, and HT29) but not in those with MIN (SW38, DLD-1, and HCT116) (Fig. 7 E). CIMD occurred in 70–90% of the tumor cells with CIN that were TUNEL + (unpublished data).

We did not detect any caspase (caspases 1, 3–9) activity in mitotic tumor cells with CIN (Fig. S4 D), and caspase inhibitors BAF and zVAD did not inhibit DNA fragmentation (Fig. S4 E). These results suggest that the tumor cell lines with CIN have defective BUB1 pathways. In an early study, BUB1 mutations were not found in these cells (Cahill et al., 1998). Therefore, we measured the BUB1 protein levels in tumor cells with CIN; the level of BUB1 expression in tumors with CIN was lower than that in tumor cells with MIN or in HeLa cells (Fig. 7 F). The BUB1 levels in the tumor cells with CIN were ~40% of that in HeLa cells (Fig. S4 F). Partial reduction of BUB1 in HeLa cells can induce CIMD; therefore, the low level of BUB1 expression could explain why the tumors with CIN induce CIMD. To test this theory, we overexpressed BUB1 in tumor cells with CIN to see whether restoring BUB1 levels suppresses CIMD. As expected, overexpression of BUB1 suppressed CIMD in the colon tumor cell lines with CIN (Fig. 7 G; Fig. S1 B). Furthermore, the expression of the Bub1 mutant allele Bub1+V400, which was found in a tumor cell with CIN, (Cahill et al., 1998) induced CIMD in HeLa cells (Fig. 7 H; Fig. S1 B). These findings suggest that CIMD is a main mechanism by which microtubule inhibitors and 17-AAG kill tumor cells with CIN.

Discussion

When the spindle checkpoint detects defects in the attachment of microtubules, it induces mitotic delay (Rieder and Maiato, 2004). The loss of the spindle checkpoint activity, especially in cells with chromosome segregation defects, is thought to result in aneuploidy. The apoptosis of aneuploid cells during the subsequent G1 phase may prevent tumorigenesis (Mollinedo and Gajate, 2003). We found that CIMD occurs in BUB1-deficient cells that have defects in kinetochore–microtubule attachment. When BUB1 is completely depleted, premature mitotic exit occurs rather than CIMD.

CIMD, mitotic catastrophe, and apoptosis

The combination of cell damage and deficient cell cycle checkpoints, in particular the DNA structure checkpoints and the spindle checkpoint, cause mitotic catastrophe (Castedo et al., 2004; Okada and Mak, 2004). Multinucleate and giant cells that contain uncondensed chromosomes form after mitotic catastrophe (Okada and Mak, 2004); these features obviously differ from those observed during CIMD and are rather similar to those observed in MAD2-depleted cells. Two types of mitotic catastrophe have been defined: in the first type, the cell dies in a p53-dependent manner during or near metaphase; in the second type, death occurs in a partially p53-dependent manner after failed mitosis and during the activation of the polyploidy checkpoint (Castedo et al., 2004). Mitotic catastrophe also is accompanied by chromatin condensation, mitochondrial release of proapoptotic proteins, caspase activation, and DNA degradation (Castedo et al., 2004). In contrast, CIMD occurs in a p53-independent manner. Furthermore, the spindle checkpoint is required for mitotic catastrophe induced by DNA-damaging agents (Nitta et al., 2004). Therefore, we conclude that the features of CIMD differ from those of mitotic catastrophe.

Several reports have described apoptosis during mitosis. High concentrations of paclitaxel (>25 nM for HeLa cells) are very cytotoxic (Woods et al., 1995). When 100–200 nM paclitaxel was used, p53-independent, TUNEL + cells appeared to be in prophase. The morphology of the TUNEL + cells also resembled that of cells undergoing apoptosis (i.e., the nuclei were bubble-shaped and fragmented); this appearance differs from that observed during CIMD. High concentrations of paclitaxel induce apoptosis by activating kinase pathways that include Akt and mTOR (Asnaghi et al., 2004). In our study, we used 10 nM paclitaxel at a low concentration (10 nM; see a comparison of the effects of different paclitaxel doses in Fig. S1 C) that was sufficient to cause substantial mitotic delay but did not induce apoptosis in most cells (Woods et al., 1995). Several hours after treatment with paclitaxel or nocodazole, 80% of Snk/Pik2-depleted p53+ cells contain active caspase 3, and 4N cells are cyclin B - (Burns et al., 2003). Mitosis/CENP-F depletion induces premature chromosome decondensation followed by cell death with caspase activation (Yang et al., 2005).

Cells depleted of hNuf2 exit directly from prolonged mitotic arrest, exhibit apoptotic cell morphology, and contain DNA that resembles DNA in cells undergoing apoptosis (DeLuca et al., 2002). Because hNuf2 depletion blocks stable kinetochore–microtubule attachment, one could argue that hNuf2 depletion–induced cell
death occurs downstream of BUB1 depletion. However, if that was the case, BUB1 depletion should cause substantial CIMD, which it does not. Also, BUB1 is not required for the kinetochore localization of hNu2 (Merardi and Sorger, 2005). Therefore, hNu2 depletion-induced cell death is unlikely to be CIMD, although further investigation is required. Neither the above-mentioned studies nor the previously described mitotic cell death findings exemplify the CIMD detected by the TUNEL assay during early mitosis and is independent of caspases and p53.

Is CIMD a type of apoptosis?
Some think that apoptosis is a type of cell death that involves caspases (Chipuk and Green, 2005), then CIMD is not a type of apoptosis. However, some think that apoptosis is defined as a physiologic “cell suicide” program characterized by lethal DNA fragmentation (Okada and Mak, 2004). Because mitotic chromosomes are fragmented during CIMD, the cells can no longer survive. Although TUNEL+ chromosomes are observed, there is no apparent reason for the cells to die immediately, unless the DNA becomes fragmented (e.g., only 5% of TUNEL+ metaphase cells had misaligned chromosomes [unpublished data]). Also, this DNA fragmentation occurs rapidly (within 20 min) after the kinetochore–microtubule attachment is disrupted (when the checkpoint fails, death should occur immediately because the checkpoint stops the cell cycle). Therefore, there must be an “active” cell death system to induce DNA fragmentation in the cells that are depleted of BUB1 and have defects in kinetochore–microtubule attachment during mitosis.

AIF and EndoG play important roles in caspase-independent cell death and are released from the mitochondria during CIMD activation. Depletion of EndoG and AIF suppressed DNA fragmentation and rescued the lethality caused by CIMD. Suppression
of DNA fragmentation by depletion of AIF was weaker than that by depletion of EndoG, probably because AIF digests DNA into large molecules (50–100 kb) (Susin et al., 1999).

On the basis of these results, we conclude that CIMD is a previously uncharacterized type of active cell death. We did not observe cytochrome c release from the mitochondria during CIMD, which is consistent with CIMD being caspase independent (Cregan et al., 2004).

Our TEM analyses revealed that after CIMD, cells contain increased numbers of abnormal mitochondria, which are seen in apoptotic cells, and autophagosomes, especially in 17-AAG–treated and BUB1-depleted cells. Autophagy promotes cell death when cells are triggered to die, but authentic apoptosis cannot occur (Eskelinen, 2005). Therefore, our TEM analyses support that CIMD is an active cell death. Substantial numbers of holes or vesicles were observed in cells after CIMD occurred, but we do not know how they were generated. Autophagy may generate the holes or vesicles via the autophagy/autolysosome pathway.

Mitotic death pathways

On the basis of our findings and those of others, we propose a model of BUB1 and MAD2 function in the spindle checkpoint pathway to determine the fate of cells with mitotic errors (Fig. 8 A). BUB1 binds kinetochores in mammalian cells before BUBR1 or MAD2 does, and BUB1 is required for the subsequent localization of CENP-F, BUBR1, CENP-E, and MAD2 (Johnson et al., 2004). These findings and ours suggest that BUB1 functions upstream of MAD2 in the checkpoint pathway. When kinetochore–microtubule attachment is defective and BUB1 function is partly altered, cells still undergo arrest in mitosis, but CIMD occurs. Thus, the full activity of BUB1 is required to protect cells from CIMD.

Many tumor cells have a diminished, but not absent, spindle checkpoint response (Kops et al., 2005). In fact, we found that in tumors with CIN BUB1 expression levels were altered, which induced CIMD, and expression of a Bub1 mutant derived from a tumor with CIN also induced CIMD. One could argue against CIMD being a cellular mechanism that guards against aneuploidy, because CIMD-inducing conditions appear to be rare. However, CIMD occurs in a low percentage of untreated BUB1-depleted cells. Also, CIMD is induced by cold shock (23°C), which is a very common stress in nature. Therefore, we speculate that CIMD is an alternative death pathway that protects cells from aneuploidy and probably from tumorigenesis. This hypothesis should be examined using the mouse model system in vivo.

To evaluate the model that BUB1 determines the fate of cells with defects in kinetochore–microtubule attachment upstream of MAD2, we performed double depletion of BUB1 and MAD2. Approximately 80% of mitotic cells that are BUB1 and MAD2 depleted showed CIMD (Fig. 8 B), and depletion of BUB1 inhibited premature mitotic exit induced by depletion of MAD2 partly (Fig. S4 G), which supports the hypothesis that BUB1 functions earlier than does MAD2.

The mitotic function of p73

The p53-related p73 proteins regulate the development of the central nervous system and the immune system. They also mediate the cell cycle and apoptosis in response to DNA damage (Ramadan et al., 2005; Coates, 2006). At the G2/M transition, p73 is phosphorylated at Thr-86 by the p34cdc2/cyclin B complex (Fulco et al., 2003). This M phase–specific phosphorylation of p73 generally hinders its transcriptional activity. However, p73-specific transcriptional targets during mitosis such as the cyclin-dependent kinase inhibitor Kip2/p57 exist (Merlo et al., 2005). Therefore, CIMD may be induced by p73-specific transcriptional target proteins during mitosis. DNA microarray analyses should be performed to identify these genes.

MAD2 depletion

The synthetic lethality caused by MAD2 siRNA and 17-AAG or paclitaxel was higher than that induced by BUB1 siRNA, which suggests that, rather than apoptosis or CIMD, another mechanism of death (e.g., reproductive death) occurs in cells treated with 17-AAG or paclitaxel and MAD2 siRNA. Although defects occur
in the kinetochore of 17-AAG–treated cells, MAD2-depleted cells do not arrest in mitosis because of defects in spindle checkpoint activity. Therefore, abnormal chromosome segregation is expected to occur and result in aneuploidy because of the premature exit from mitosis. In fact, the number of abnormal nuclei, including micronuclei, was increased substantially in 17-AAG–treated and MAD2-depleted cells but not in 17-AAG–treated and BUB1-depleted cells. Although these phenotypes are more drastic, they resemble those of MAD2- or BUBR1-depleted cells (Kops et al., 2004). Therefore, mitotic catastrophe–like death that is presumably caused by premature exit from mitosis appears to occur in most MAD2-depleted and 17-AAG– or paclitaxel-treated cells.

Sudo et al. (2004) suggested that inactivation of MAD2 increases cell survival upon paclitaxel treatment (Sudo et al., 2004). This finding is inconsistent with ours. We believe that this difference can be explained by Sudo et al.’s use of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and trypan blue to evaluate cell viability, as neither of those methods detect reproductive cell death.

**Survivin**

The inhibitor of apoptosis protein survivin is also known as a chromosome passenger protein that associates with kinetochores transiently and is required for the spindle checkpoint function (Li et al., 1998; Skoutias et al., 2000; Carvalho et al., 2003; Lens et al., 2003). Disruption of survivin–microtubule interactions inhibits the anti-apoptosis function of survivin and induces caspase-3 activity (Li et al., 1998). Survivin regulates mitochondrial apoptosis and caspase-9 recruitment to the Apaf-1 apoposome (Marusawa et al., 2003). Therefore, survivin does not appear to be involved in CIMD. Overexpression of survivin does not suppress CIMD (Fig. S5, A and B, available at http://www.jcb.org/cgi/content/full/jcb.200702134/DC1), though it does suppress paclitaxel- or geldanamycin-induced, caspase-mediated apoptosis (Li et al., 1998; Fortugno et al., 2003).

**EndoG-null mice**

Although EndoG was initially shown to be required for early embryogenesis and normal apoptosis in mice (Zhang et al., 2003), recently reports in EndoG-null mice have shown that EndoG is dispensable in embryogenesis and apoptosis (Irvin et al., 2005; David et al., 2006). However, the results from these reports do not exclude the involvement of EndoG in CIMD nor deny the potential significance of CIMD in antitumorogenesis. We showed that EndoG is required for CIMD but redundantly with AIF. *Bub1* transgenic mice did not show increased predisposition to spontaneous tumors (Cowley et al., 2005), raising the possibility that CIMD protects these animals from tumorigenesis. Further investigation is needed to address the function of CIMD in mice with multiple mutations of key factors.

**CIMD of tumor cells with CIN treated with microtubule inhibitors or 17-AAG**

Successful Phase I trials of 17-AAG have recently been completed; 17-AAG was well tolerated at doses that modulate the level of Hsp90 client proteins (Banerji et al., 2005; Glaze et al., 2005; Goetz et al., 2005; Grem et al., 2005; Ramanathan et al., 2005). CIMD may be a main mechanism by which 17-AAG and microtubule inhibitors kill tumor cells with CIN. The similarity between the cell death response induced by 17-AAG and that induced by microtubule inhibitors, which are classic anti-cancer agents (Mollinedo and Gajate, 2003), suggests that similar mechanisms are involved, although 17-AAG uses multiple mechanisms (Miyata, 2005).

Last but not least, the mitotic index has been used to evaluate spindle checkpoint activity. Our findings suggest that previous studies probably failed to detect defects in the BUB1 pathway, because the mitotic index appears to be normal during CIMD. Thus, the results of those earlier studies, especially those involving tumor cells, should be reevaluated with consideration of the occurrence of CIMD. Furthermore, the detection of CIMD could be a novel method for diagnosing tumors with CIN.

**Materials and methods**

**siRNA**

The siRNAs targeting MAD2 and luciferase have been described previously (Elbashir et al., 2001; Martin-Ueema et al., 2002). We used three BUB1 siRNAs: 5′-GGCUUGCAACCCCGUGGAAAT-3′ (BUB1 siRNA#1), 5′-CAAC-ACUUAUCUAACAGAAT-3′ (BUB1 siRNA#2), and 5′-CCAGGCUGAAC-CAGAGATG-3′ for the studies described in the main text. Similar data were obtained when these independent sets of siRNAs were used. The siRNA targeting AIF has been described previously (Bidere et al., 2003). We also designed another AIF siRNA: 5′-CUGUUCACCAGGCAUGCAUUU-3′. Similar data were obtained when these two independent sets of siRNAs were used. We designed three sets of EndoG siRNAs: 5′-AAGAGCAGCCGAGCGUA-CGU-3′, 5′-AAAGCACCUGUGAGAGGCC-3′, and 5′-CGGGCCUGGG-GCCUCGUCUUU-3′, and similar data were obtained when these three independent sets of siRNAs were used. The siRNAs targeting MAD2, BUB1, AIF, and EndoG were synthesized by the Hartwell Center for Bioinformatics and Biotechnology at St. Jude Children’s Research Hospital (Memphis, TN).

**Antibodies**

Table S2 lists the antibodies used in this study (available at http://www.jcb.org/cgi/content/full/jcb.200702134/DC1).

**Colony outgrowth assay**

The colony outgrowth assay was performed as described previously (Blasco et al., 1997; Reilly et al., 2002; Kranc et al., 2003) with a minor modification. Hela cells were transfected with siRNAs by using Lipofectamine 2000. 24 h after transfection, the cells were incubated with DME (BioWhittaker) with 10% fetal bovine serum (FBS; Invitrogen); Caco-2 cells were incubated in high glucose DMEM (BioWhittaker) with 10% fetal bovine serum (FBS; Invitrogen); Caco-2 cell death detection system that contained TMR red (Roche).

**TUNEL assay**

48 h after siRNA transfection, Hela cells, tumor cells with CIN, or tumor cells with MIN were incubated with 500 nM 17-AAG (A.G. Scientific) for 24 h. Cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (pH 7.4), and the TUNEL assay was performed by using an in situ cell death detection system that contained TMR red (Roche).

**Caspase assay**

Hela cells were transfected with siRNA, and 48 h later the cells were incubated in 500 nM 17-AAG for 24 h. The FLICA caspase assay was performed by using the carboxyfluorescein FLICA (Poly-Caspases FLICA [FAM-VAD-FMK]) apoptosis detection system (Immunochemistry Technologies, LLC).

**Cell culture and transfection**

All human cell lines were purchased from American Type Culture Collection (Manassas, VA). Hela and SW480 cells were cultured in high glucose DMEM (BioWhittaker) with 10% fetal bovine serum (FBS; Invitrogen); Caco-2

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and RKO cells, in Eagle’s minimum essential medium (ATCC) with 10% FBS; HT29 and HCT116 cells, in McCoy’s 5A medium (ATCC) with 10% FBS; and DLD-1 cells, in RPMI-1640 medium (ATCC) with 10% FBS. All cell lines were grown at 37°C in 5% CO2 in a humidified incubator. Cells were transfected with annealed double-stranded siRNA or mammalian expression plasmids by using Lipofectamine 2000 (Invitrogen) or Fugene 6 (Roche).

Immunoblotting
The method of immunoblotting has been described in detail elsewhere (Lamb et al., 1995; Kitagawa et al., 1999). Cells were added to lysis buffer A (Panaretou et al., 2002), and the mixture was frozen in liquid nitrogen, thawed, and sonicated. Before electrophoresis, cell lysates were mixed with an equal volume of 2× SDS sample buffer.

Immunofluorescence
Methods of indirect immunofluorescent staining have been described previously (Tugendreich et al., 1995; Yoda et al., 1996), but were slightly modified. HeLa cells were grown for 48 h on coverslip slides ( seeding, ~2.0 × 104 cells/Well). Asynchronous cells were fixed briefly in 4% paraformaldehyde in phosphate-buffered saline at 4°C for 30 min and then treated with 0.5% Triton X-100 in KB [10 mM Tris HCl, pH 7.5, 150 mM NaCl, and 0.5% bovine serum albumin] at room temperature for 30 min. The cells were then incubated with a specific primary antibody for 1 h at 37°C. After the cells were washed once with KB, they were incubated with the fluorescent secondary antibodies fluorescein isothiocyanate-conjugated Alexa Fluor 488 (Invitrogen), Texas red-conjugated AlexaPure IgG (Jackson ImmunoResearch Laboratories) for 1 h at 37°C. Slides were washed once with KB and then incubated in KB containing 0.1 μg/ml DAPI (Sigma-Aldrich). Cells were observed through an Axioskop2 (Carl Zeiss MicroImaging, Inc.) motorized fluorescence microscope equipped with a Plan Achromat 63× oil immersion lens (Carl Zeiss MicroImaging, Inc.), an HBO 100 microscope illuminator (Aloca), and a microMAX CCD camera (Princeton Instruments, Inc.). Appropriate filters were used to photograph stained cells. Image acquisition and processing was performed with IP Lab Scientific Imaging Software (Scanalytics). Alternatively, we observed cells through a DM IRE2 motorized fluorescence microscope (Leica) equipped with an HCX PL APO 63× oil immersion lens (Leica), an ARC LAMP power supply HBO100 DC ICG (Ludl Electronic Products, Ltd.), and an ORCA-ER high-resolution digital CCD camera (Hamamatsu). Image acquisition and processing were performed using Openlab version 4 Scientific Imaging Software (Improvision).

DNA fragmentation assay
A DNA fragmentation assay was performed as described previously (Sonoda et al., 1997, 1999, 2000). In brief, cells were gently lysed for 30 min at room temperature in buffer containing 5 mM Tris HCl (pH 7.4), 20 mM EDTA, and 0.5% Triton X-100. After centrifugation at 15,000 rpm for 15 min, supernatants containing soluble, fragmented DNA were collected and treated with RNase (20 μg/ml; Sigma-Aldrich) and then with protease K (20 μg/ml). DNA fragments were precipitated in 99% ethanol. Samples were then subjected to electrophoresis in a 2% agarose gel and visualized by staining with ethidium bromide.

Time-lapse imaging and analysis
HeLa cells were plated on 10-mm-diameter tissue culture plates with glass bottoms (MatTek Corp.) that had been coated with poly-lysine. Cells were transfected with either human BUB1 siRNA or luciferase siRNA using Lipofectamine 2000 (Invitrogen). After 48–54 h, cells were incubated with 1 mM ICRF187 (cardioxane; Chiron Corp.). After 54 h, cells were transferred to L15 Leibovitz medium to which 2.05 mM L-glutamine (HyClone) had been added. The medium was then supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin (both from Invitrogen). At the same time cells were coincubated with 1 mM ICRF187 and 500 nM 17-AAG, which were added to the cells. Cells were maintained at 37°C. Phase-contrast images were captured every half hour for 24 h (after 54–78 h of transfection). Cells were then transfected with either human BUB1 siRNA or luciferase siRNA using Lipofectamine 2000 (Invitrogen) and treated with RNase (20 μg/ml; Sigma-Aldrich) and then with protease K (20 μg/ml). DNA fragments were precipitated in 99% ethanol. Samples were then subjected to electrophoresis in a 2% agarose gel and visualized by staining with ethidium bromide.

Electron microscopy
Mitotic HeLa cells were collected by gentle pipetting and fixed briefly with a 37°C solution of 2% paraformaldehyde, 2.5% glutaraldehyde in 0.15 M sodium cacodylate (pH 7.4). Low-melting point agarose (0.5%) was mixed with 0.1 M cacodylate buffer, pH 7.4, and then poured on glass slides that were coated with Sigma mineral oil. Cells were then main-
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