Cancer Cell
Article

Evidence that Mitotic Exit Is a Better Cancer Therapeutic Target Than Spindle Assembly

Hsiao-Chun Huang,[1,2,*] Jue Shi,[1,3] James D. Orth,[1] and Timothy J. Mitchison[1]
[1]Department of Systems Biology, Harvard Medical School, Boston, MA 02215, USA
[2]Graduate Program in Systems Biology, Harvard Medical School, Boston, MA 02215, USA
[3]Department of Physics, Hong Kong Baptist University, Kowloon Tong 852, Hong Kong
*Correspondence: hsiao-chun_huang@hms.harvard.edu
DOI 10.1016/j.ccr.2009.08.020

SUMMARY

Current antimitotics work by perturbing spindle assembly, which activates the spindle assembly checkpoint, causes mitotic arrest, and triggers apoptosis. Cancer cells can resist such killing by premature exit, before cells initiate apoptosis, due to a weak checkpoint or rapid slippage. We reasoned blocking mitotic exit downstream of the checkpoint might circumvent this resistance. Using single-cell approaches, we showed that blocking mitotic exit by Cdc20 knockdown slowed cyclin B1 proteolysis, thus allowed more time for death initiation. Killing by Cdc20 knockdown did not require checkpoint activity and can occur by intrinsic apoptosis or an alternative death pathway when Bcl2 was overexpressed. We conclude targeting Cdc20, or otherwise blocking mitotic exit, may be a better cancer therapeutic strategy than perturbing spindle assembly.

INTRODUCTION

Antimitotic drugs that target microtubule dynamics, including taxanes, vinca alkaloids, and epothilones, are active against a broad range of cancers, but they also cause neurotoxicity, presumably due to perturbation of microtubules in neurons (Jordan and Wilson, 2004). In an effort to develop antimitotic drugs lacking this toxicity, small-molecule inhibitors of a number of proteins specific to the mitotic spindle were developed, including the motor protein kinesin-5 (KSP, Eg5, Kif11), Aurora kinases, and Polo-like kinases (Jackson et al., 2007). In clinical trials to date, these spindle-specific antimitotic drugs lack neurotoxicity as hoped, but their efficacy against solid tumors seems to be no better than taxanes and vincas, and perhaps not as good. Can we find an antimitotic strategy that not only lacks neurotoxicity, but is also more effective than current strategies at causing regression of solid tumors? We set out to address this question using RNAi knockdown as a surrogate for potential drugs, and comparing efficacy for killing cancer cell lines with representative drugs that interfere with spindle assembly.

The net effect of antimitotic drugs is to perturb mitotic spindle assembly, which activates the spindle assembly checkpoint (SAC). After many hours of SAC-induced mitotic arrest, cancer cells either die inside mitosis, or exit mitosis by slippage into a tetraploid G1 state, from which they either die, arrest in G1, or initiate a new round of the cell cycle (Rieder and Maiato, 2004; Gascoigne and Taylor, 2008; Orth et al., 2008). Slippage is thought to occur by gradual proteolysis of cyclin B1, which continues slowly even when the SAC is active (Brito and Rieder, 2006). Cell death occurs mainly via activation of the intrinsic apoptosis (Wang et al., 1999; Park et al., 2004; Tao et al., 2005; Bergstralh and Ting, 2006), a pathway involving mitochondrial outer membrane permeabilization (MOMP) (Letai, 2008). Failure to initiate apoptosis during or after mitotic arrest appears to be a major factor limiting efficacy of antimitotic drugs because mitotic arrest without subsequent apoptosis is commonly observed following taxane treatment in various cancer cell lines (Shi et al., 2008), mouse cancers (Milross et al., 1996), and, though data are very limited, human breast cancers, where it correlates with poor tumor responses (Symmans et al., 2000).

SIGNIFICANCE

Drugs targeting spindle proteins, including kinesin-5, Aurora kinases, and Polo-like kinases, were recently developed in the hope of providing less neurotoxic antimitotic drugs. In clinical trials to date, their efficacy seems no better than taxanes and vincas. We provide evidence that an alternative antimitotic strategy, blocking mitotic exit, might be more effective. We found Cdc20 knockdown provides a checkpoint-independent mitotic arrest that kills cancer cells more effectively than spindle-perturbing drugs. We suggest mitotic exit is a promising therapeutic target, and propose a cell-based screening strategy for compounds with this mechanism. These findings are significant from a drug development perspective, and also as a mechanistic step toward understanding how mitotic arrest triggers death, and how some cancer cells resist this trigger.

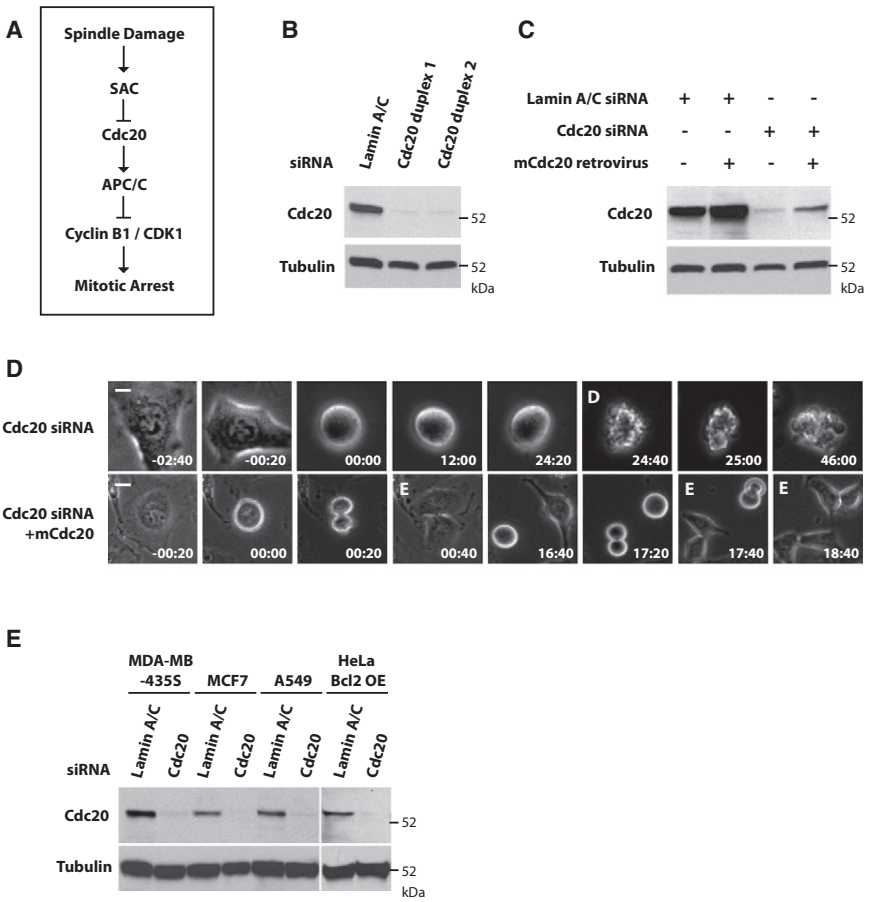

Figure 1. Cdc20 as a Potential Antimitotic Drug Candidate

(A) Simplified pathway from spindle damage to mitotic arrest.

(B) siRNA knockdown of Cdc20 in HeLa cells as analyzed by immunoblotting for Cdc20 and tubulin.

(C) Rescue of Cdc20 knockdown by expression of an RNAi-resistant transgene to confirm the specificity of siRNA. HeLa cells infected with retrovirus expressing control vector (–) or mouse Cdc20 cDNA with two extra silent mutations (mCdc20) were transfected with control Lamin A/C (–) or Cdc20 siRNA. Cell lysates were immunoblotted for Cdc20 or tubulin.

(D) Phase-contrast time-lapse analysis of progression through mitosis and death in HeLa cells under conditions indicated at left. Numbers show elapsed time (hour:minute) relative to mitotic entry. Time of death (to the nearest frame) is indicated as D, and mitotic exit is indicated as E. The scale bar represents 5 μm.

(E) siRNA knockdown of Cdc20 in MDA-MB-435S, MCF7, A549, and HeLa Bcl2-overexpressing cells as analyzed by immunoblotting for Cdc20 and tubulin.

Here, we focus on drug resistance caused by lack of apoptosis downstream of spindle damage; clinical resistance might also arise from mutations that prevent drugs from causing spindle damage, e.g., due to target protein mutations or drug efflux pump expression (Pusztai, 2007), from failure of cancer cells to enter mitosis during drug exposure (Baguley et al., 1995), or other causes.

Previous studies provide two mechanistic clues to how cancer cells choose a nonapoptotic outcome following spindle damage and mitotic arrest. First, they may fail to execute apoptosis efficiently due to downregulation of apoptosis pathways. Protection against MOMP at the level of Bcl2 protein family reduces sensitivity to apoptosis promoted by paclitaxel and vinca alkaloids (Tan et al., 2005; Deng et al., 2007; Kutuk and Letai, 2008). Second, they may slip out of mitotic arrest before they die; in other words, slippage and apoptosis can be viewed as two competing pathways (Gascoigne and Taylor, 2008). Consistent with slippage protecting cells from death, premature exit from mitotic arrest due to a weakened or ablated SAC is known to decrease sensitivity to spindle-perturbing drugs (Taylor and McKeon, 1997; Shin et al., 2003; Tao et al., 2005; Swanton et al., 2007; Gascoigne and Taylor, 2008; Bekier et al., 2009). Based on these clues, we reasoned that blocking mitotic exit downstream of the SAC may be a better strategy for killing apoptosis-resistant, slippage-prone, or SAC-defective cancer cells than any of the currently available antimitotic drugs, all of which target spindle assembly.

RESULTS

Cdc20 Knockdown Causes Mitotic Arrest and Cell Death

As surrogate for a potential drug that directly blocks mitotic exit, we knocked down Cdc20 using siRNAs. Cdc20 activates the APC/C to trigger cyclin B1 degradation during normal mitosis, and it is sequestered by SAC proteins when the spindle is damaged (Figure 1A) (Musacchio and Hardwick, 2002; Musacchio and Salmon, 2007). Cdc20 must be depleted to less than 5% of its normal levels to arrest cells in mitosis (Wolthuis et al., 2008). We tested several siRNA duplexes and hairpin constructs in HeLa cells, and selected two duplexes on the basis of promoting the most robust mitotic arrest, and most efficient knockdown by immunoblotting (Figure 1B). All data shown are for duplex 1, but similar results were obtained using duplex 2. HeLa cells depleted of Cdc20 arrested in mitosis for an average of 18.8 ± 7.3 hr (n = 98) before undergoing death in mitosis (Figure 1D, top panel). Specificity is a major concern for siRNA duplexes; to evaluate this, we performed a RNAi-resistant transgene rescue experiment for duplex 1, using mouse Cdc20 cDNA with two extra silent mutations (mCdc20) as the rescue construct (Figure 1C). In HeLa cells infected with control vector, and transfected with duplex 1, more than 98% underwent prolonged arrest followed by death in mitosis. In cells infected with retrovirus expressing mCdc20, and then transfected with duplex 1, 83% went through mitosis with little or no delay (1.5 ± 0.9 hr, n = 85), divided, did not die, and continued to the next cell cycle (Figure 1D, bottom panel). The remaining 17% that still showed prolonged arrest may not have been infected with the rescue construct. We conclude that the robust arrest and cell death phenotype caused by duplex 1 is specific to knockdown of

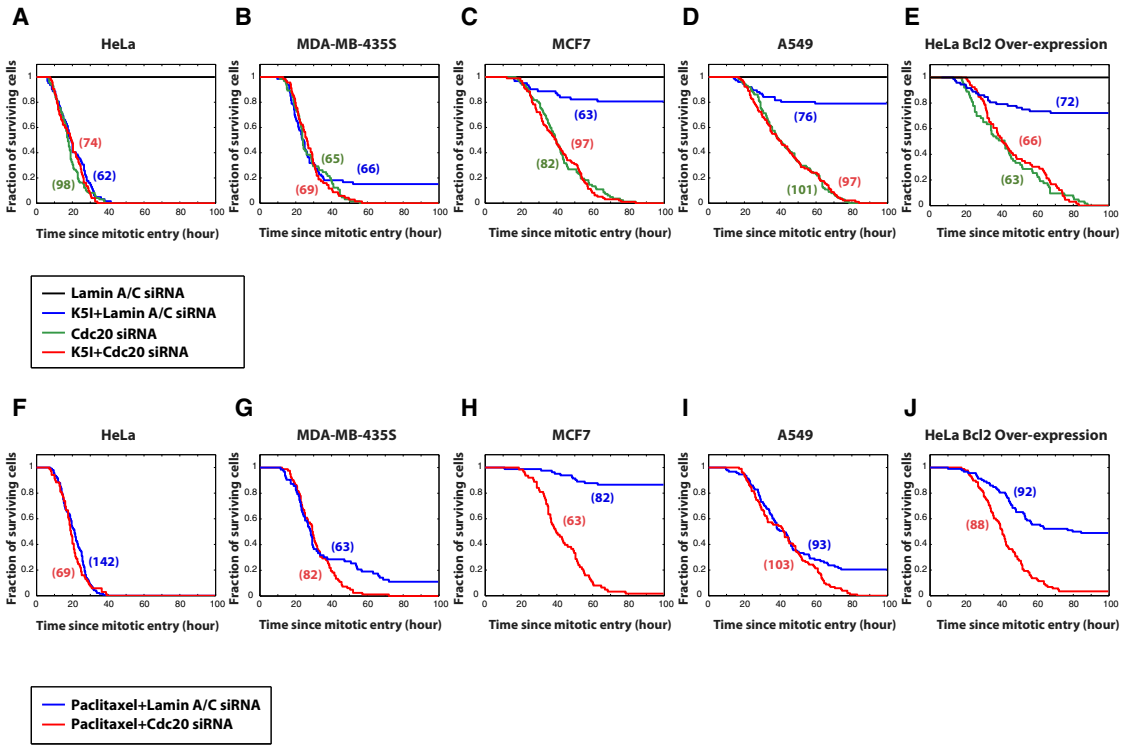

Figure 2. Knockdown of Cdc20 Efficiently Kills Slippage-Prone and Apoptosis-Resistant Cancer Cells

Cumulative survival curves for indicated treatments in various cancer cell lines: HeLa (A and F), MDA-MB-435S (B and G), MCF7 (C and H), A549 (D and I), and HeLa cells stably expressing Bcl2 (E and J). Individual cells were monitored by phase-contrast time-lapse microscopy, and time from mitotic entry to death was measured and plotted as survival curves as a function of time since mitotic entry. For Cdc20 siRNA transfection alone, only cells that entered and underwent > 6 hr mitosis (varying from 30%–100%) are considered to be Cdc20 knocked down and included in our analysis. For all other treatments, all cells that entered mitosis are analyzed. When cell division occurred, daughter cells were counted as one. Numbers in parentheses indicate number of cells scored.

Cdc20. Duplex 1 also efficiently knocked down Cdc20 in four other cell lines we investigated below (Figure 1E).

Cdc20 Knockdown Efficiently Kills Slippage-Prone and Apoptosis-Resistant Cancer Cells

We next systematically compared the ability to promote death during mitotic arrest between Cdc20 knockdown and treatment with a mitosis-specific kinesin-5 inhibitor, EMD534085 (Orth et al., 2008). We made this comparison in five solid-tumor-derived cell lines: four were selected from a larger panel tested previously so as to span the full range of death sensitivity when treated with antimitotic drugs (Shi et al., 2008); Bcl2-overexpressing HeLa cells were added as a fifth line with a known mechanism of apoptosis resistance. Because individual cells vary greatly in their kinetics of mitotic arrest and death during mitosis (Gascoigne and Taylor, 2008; Orth et al., 2008), we quantified single-cell behavior using time-lapse microscopy. Figures 2A–2E show death kinetics in individual cells by time-lapse phase-contrast imaging, where death was scored by vigorous blebbing followed by cessation of all movement. Time of death was normalized to time of mitotic entry, which was scored by cell rounding. Because both kinesin-5 and Cdc20 are thought to function only in mitosis, and death in both kinesin-5 inhibitor and Cdc20 knockdown only occurred during or after mitotic arrest, normalizing so that T = 0 was the time of mitotic entry conceptually synchronizes all cells at the start of the prodeath stimulus. These data compare four treatments: lamin A/C siRNA alone (to control for the toxicity of siRNA transfection), kinesin-5 inhibitor plus lamin A/C siRNA, Cdc20 siRNA, and kinesin-5 inhibitor plus Cdc20 siRNA. A saturating concentration of kinesin-5 inhibitor was used, so all drug-treated cells that entered mitosis arrested, and none succeeded in executing cytokinesis. For kinesin-5 inhibitor treatment, we observed some death in mitosis, some slippage, and some death after slippage in all lines. These data are reported separately in Table 1. For simplicity, Figures 2A–2E report kinetics of all death, whether it occurred before or after slippage, as cumulative survival curves. For Cdc20 knockdown, we observed no slippage. HeLa was the most death sensitive in our previous profiling experiment (Shi et al., 2008). In this line, more than 90% of cells died during mitotic arrest for all treatments except control siRNA alone, and death kinetics were similar in each case (Figure 2A). In moderately resistant MDA-MB-435S, 15% cells slipped out of kinesin-5 inhibitor-induced mitotic arrest and survived, and in highly resistant MCF7 and A549, ~80% slipped and survived (blue lines in Figures 2B, 2C, and 2D; Table 1). In each of these lines, knockdown of Cdc20 prevented slippage, regardless of whether kinesin-5 inhibitor was present. All Cdc20 knocked-down cells remained arrested in mitosis for the whole time course, and all eventually died (red and green lines in Figures 2B, 2C, and 2D).

The molecular origin of death resistance in MCF7 and A549 is incompletely understood. To compare Cdc20 knockdown with

Table 1. Duration of Mitosis and Percentage of Cell Death in Response to Kinesin-5 Inhibitor or Paclitaxel Incubated with Lamin A/C siRNA

	Kinesin-5 Inhibitor				Paclitaxel			
Cell Line	Duration of Mitosis ± SD (hr)	Death (%)[a]	D in M (%)[b]	D after E (%)[c]	Duration of Mitosis ± SD (hr)	Death (%)[a]	D in M (%)[b]	D after E (%)[c]
HeLa	19.7 ± 7.7 (n = 62)	98.4	90.3	8.1	21.3 ± 6.8 (n = 142)	100.0	100.0	0.0
MDA-MB-435S	23.6 ± 6.3 (n = 66)	84.9	77.3	7.6	24.9 ± 7.9 (n = 63)	88.9	71.4	17.7
MCF7	19.2 ± 6.6 (n = 63)	21.0	8.1	12.9	15.7 ± 7.3 (n = 82)	13.4	2.4	11.0
A549	17.9 ± 4.0 (n = 76)	20.8	6.5	14.3	16.3 ± 4.4 (n = 93)	79.6	5.4	74.2
HeLa Bcl2 OE[d]	19.7 ± 8.8 (n = 72)	27.8	15.3	12.5	20.1 ± 7.1 (n = 92)	52.2	6.5	45.7

[a] Percentage of total cell death.
[b] Percentage of death in mitosis.
[c] Percentage of death after mitotic exit.
[d] HeLa cells overexpressing Bcl2.

kinesin-5 inhibitor in cells where we know the origin of death resistance, we used a HeLa line that stably overexpresses Bcl2. Bcl2 antagonizes MOMP, and overexpression of Bcl2 and related family members has been widely implicated in apoptosis resistance in cancer (Letai, 2008). More than 70% of HeLa cells overexpressing Bcl2 slipped out of mitotic arrest induced by kinesin-5 inhibitor, and survived (blue line in Figure 2E; Table 1), like the naturally death-resistant cancer lines. Cdc20 knockdown again prevented slippage, and killed all cells that entered mitosis (red and green lines in Figure 2E), though this took ~2.5-fold longer in time on average than normal HeLa (compare red and green lines in Figures 2A and 2E).

These data allow several conclusions. First, Cdc20 knockdown efficiently promotes death during mitotic arrest. In lines that tend to die inside mitosis in kinesin-5 inhibitor, Cdc20 knockdown is equally effective at promoting death, but in lines that tend to slip before they die, it is much more effective. Second, because Cdc20 knockdown blocks slippage, these data allow us to compare the rate of death induction during mitotic arrest among the lines, without the complication of slippage. The median times for induction of death in Cdc20 knockdown were: HeLa 18.0 hr, MDA-MB-435S 24.3 hr, MCF7 39.8 hr, A549 40.0 hr, HeLa overexpressing Bcl2 40.8 hr. Thus, death induction rates during mitotic arrest were ~2.5-fold faster in the most death-sensitive line compared with the most resistant. This relatively small difference in death induction rate translates into a much larger difference in survival in kinesin-5 inhibitor (~0% in HeLa compared with >70% in MCF7, A549 and HeLa overexpressing Bcl2) because slippage intervenes to rescue the slower-dying lines, as proposed in the competing pathway model (Gascoigne and Taylor, 2008). Finally, in HeLa cells Bcl2 overexpression confers strong resistance to kinesin-5 inhibitor, but not to Cdc20 knockdown.

We next extended the comparison to paclitaxel, a drug with proven activity in solid tumors (Jordan and Wilson, 2004). Again, we used a drug concentration that was saturating for mitotic arrest and failure of cytokinesis in all lines, to avoid complications from drug efflux pump or tubulin isotype differences. Across the panel, addition of Cdc20 knockdown to paclitaxel was always as, or more, efficient than paclitaxel alone at inducing cell death (Figures 2F–2J). In some lines, paclitaxel is more proapoptotic than kinesin-5 inhibitor. The duration of mitotic arrest was essentially the same for both drugs in all lines, and the extra cell death in paclitaxel manifested mostly after slippage (Table 1; Shi et al., 2008). In the more death-sensitive lines (HeLa, MDA-MB-435S), paclitaxel and kinesin-5 inhibitor caused death with similar kinetics, and Cdc20 knockdown killed with either the same (HeLa) or somewhat greater (MDA-MB-435S) efficiency. Death-resistant MCF7 cells responded similarly to the two drugs, and in this line Cdc20 knockdown killed with much greater efficiency than either drug. A549 cells were killed more efficiently by paclitaxel than kinesin-5 inhibitor, but Cdc20 knockdown was yet more efficient. HeLa overexpressing Bcl2 was intermediate between MCF7 and A549. Overall, although paclitaxel was somewhat more efficient at promoting killing than kinesin-5 inhibitor in some apoptosis-resistant lines, Cdc20 knockdown was always more efficient than either drug.

A priori, we do not expect Cdc20 knockdown to perturb spindle assembly or activate the SAC. To test whether Cdc20 knockdown perturbs spindle assembly, we imaged microtubules live in HeLa stably expressing green fluorescent protein (GFP) β-tubulin (Figure 3). We observed normal bipolar spindles early in the arrest, which gradually became multipolar and abnormal over hours. From these images, it seems likely that the SAC is not activated early in the Cdc20 knockdown arrest, though it may be activated later. Because combining Cdc20 knockdown and kinesin-5 inhibitor showed similar death kinetics to Cdc20 knockdown alone in all lines (red and green lines in Figures 2A–2E), we used this combination in most subsequent experiments. By deliberately activating the SAC, we removed the ambiguity of whether it was activated. Combination with drugs was also more reliable for blocking slippage than Cdc20 knockdown alone in cell lines where transfection efficiency was variable.

Cdc20 Knockdown Slows Cyclin B1 Proteolysis

To determine how Cdc20 knockdown prevents slippage, we imaged cells infected with adenovirus expressing full-length cyclin B1 fused to enhanced green fluorescent protein (EGFP) (Bentley et al., 2007). We first confirmed that our cyclin B1-EGFP expression did not affect normal mitosis, duration of drug-induced mitotic arrest, or kinetics of cell death (data not shown). In HeLa, where most cells died in mitosis in kinesin-5 inhibitor, cyclin B1 levels gradually decreased to 30%–60% of the starting value by the time of death (Figure 4A, n > 30). In A549, where

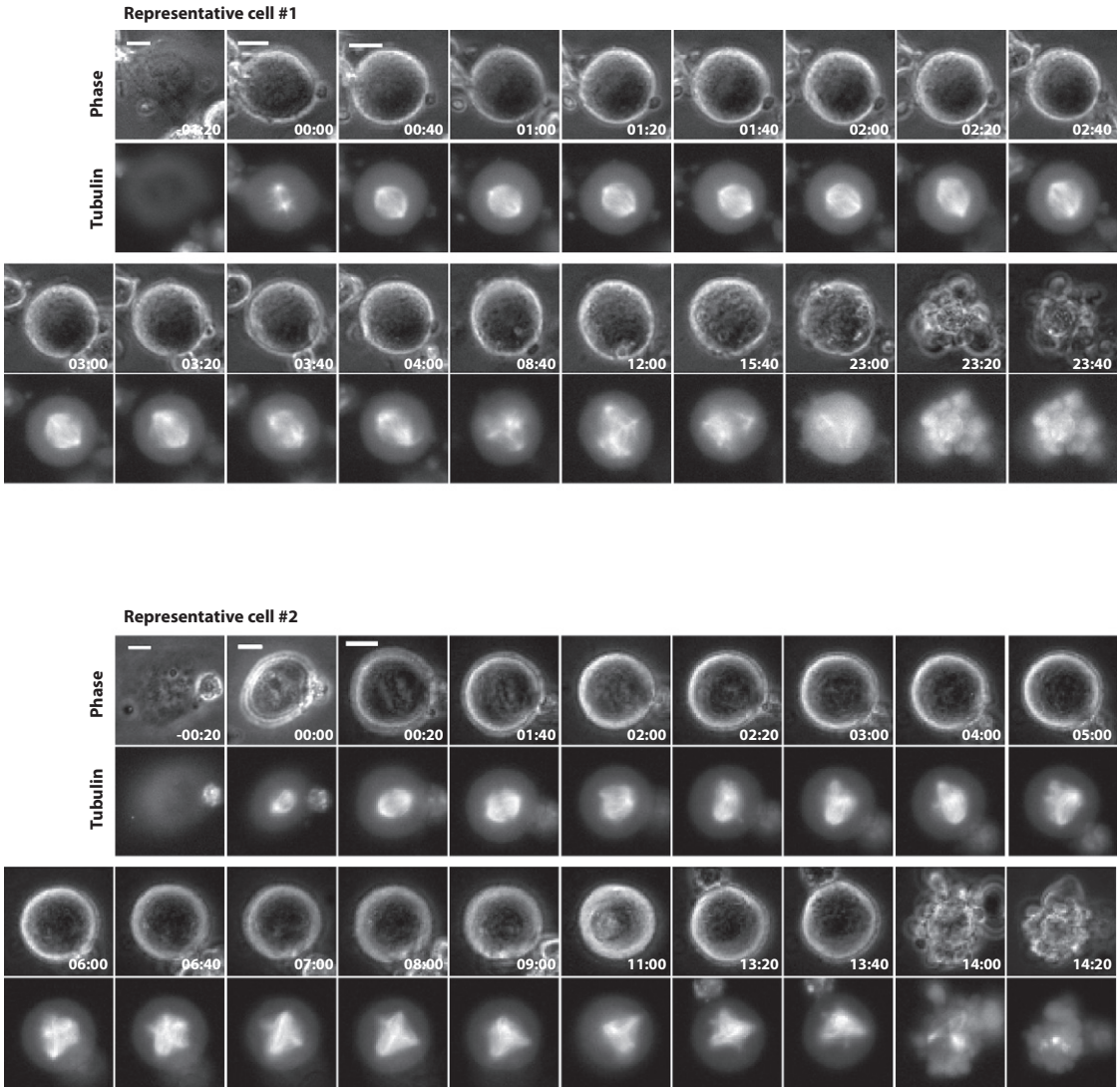

Figure 3. Knockdown of Cdc20 Induces Spindle Damage over Time

Time evolution of mitotic spindles from two representative HeLa-GFP-β-tubulin cells. Cells were transfected with Cdc20 siRNA and monitored by time-lapse microscopy. Numbers indicate elapsed time (hour: minute) relative to mitotic entry. The scale bar represents 5 μm.

most cells slipped out of arrest without dying in kinesin-5 inhibitor, cyclin B1 levels slowly decreased, until they were 0%–10% of the level at the start of mitosis, when the cell slipped by morphological criteria (Figure 4B, n > 40). We observed considerable cell-to-cell variation in the shape and slope of cyclin B1 decrease kinetics, as we might expect because slippage kinetics are highly variable from cell to cell (Gascoigne and Taylor, 2008; Orth et al., 2008), but slippage always correlated with the time that cyclin B1 levels were reduced to 0%–10% of their starting value. When Cdc20 was depleted, cyclin B1 levels declined more slowly, especially in A549 (Figures 4B and 4D). In this situation, each time course ended when the cell underwent death in mitosis, which occurred on average 18.8 ± 7.3 hr (n = 98) after mitotic entry in HeLa, and 43.8 ± 16.5 hr (n = 101) in A549. At this time, cyclin B1 levels were 50%–90% of their mitotic entry value in HeLa, and 30%–70% in A549. Similar results were found when we used HeLa and A549 lines stably expressing full-length cyclin-B1-EYFP, suggesting that such degradation kinetics is not specific to adenovirus-mediated expression of cyclin-B1-EGFP (see Figure S1 available online). We conclude that Cdc20 knockdown stabilizes cyclin B1 levels during mitotic arrest more efficiently than SAC activation via kinesin-5 inhibition. This presumably explains why arrest is sustained for longer in Cdc20 knockdown, which gives cells more time to die in mitosis. These data are also consistent with a previous hypothesis that slippage is due to slow proteolysis of cyclin B1 by leaky activity of the APC/C^{Cdc20} - proteasome pathway even when SAC is active (Brito and Rieder, 2006), though a potential complication is the recent observation that cyclin B1 turns over with a half life of 1–2 hr, so its gradual loss presumably reflects a balance between synthesis and proteolysis (Nilsson et al., 2008). Other mitotic cyclins could potentially contribute to Cdc20

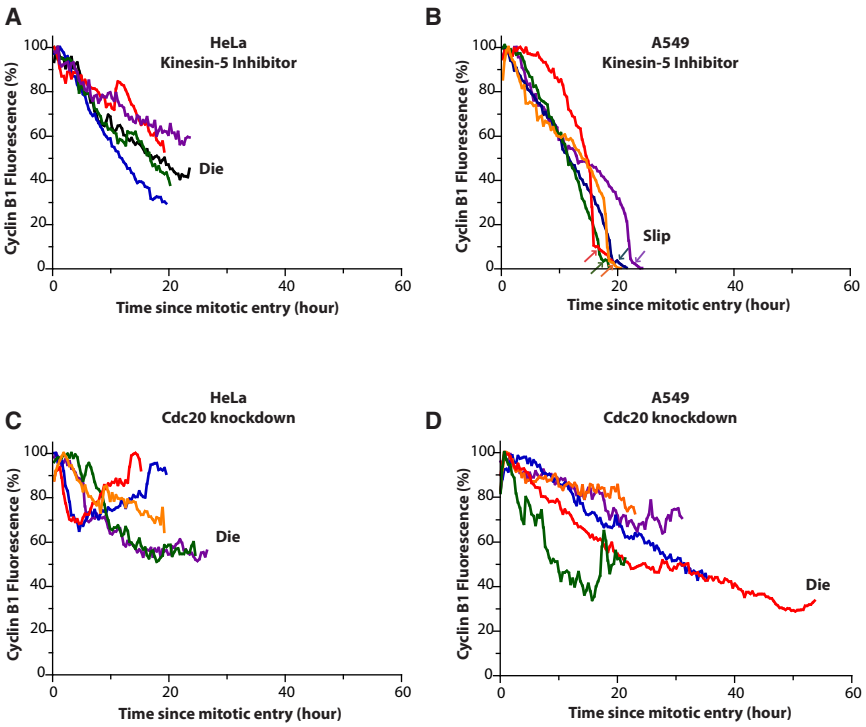

Figure 4. Knockdown of Cdc20 Slows Cyclin B1 Proteolysis

Changes of cyclin B1 level over time from five representative HeLa or A549 cells treated with kinesin-5 inhibitor or Cdc20 siRNA. Cells infected with adenovirus expressing full-length cyclin B1-GFP were treated as indicated and monitored by time-lapse microscopy. Fluorescent intensity (with subtraction of background) was normalized by the highest intensity in the time course for each cell. T = 0 marks mitotic entry.

(A) HeLa cells died in mitosis under kinesin-5 inhibitor.

(B) A549 cells slipped out of mitotic arrest induced by kinesin-5 inhibitor.

(C) HeLa cells died in mitosis when treated with Cdc20 siRNA.

(D) A549 cells died in mitosis when treated with Cdc20 siRNA.

knockdown-mediated mitotic arrest, because depletion of Cdc20 also stabilizes other APC/C^{Cdc20} substrates, for example cyclin A (Sigrist et al., 1995; Wolthuis et al., 2008).

Death Induced by Cdc20 Knockdown Does Not Depend on SAC Activity

Loss or weakening of SAC activity confers strong resistance to SAC-dependent antimitotic drugs in various cancer cell lines (Taylor and McKeon, 1997; Shin et al., 2003; Tao et al., 2005; Swanton et al., 2007; Gascoigne and Taylor, 2008; Bekier et al., 2009). To test whether Cdc20 knockdown can efficiently kill SAC-deficient cells, we knocked down individual SAC proteins in HeLa cells by siRNA transfection, testing Mad2, BubR1, Mps1, and Bub3 (Figure 5A). Mad2, BubR1, and Bub3 are present in the mitotic checkpoint complex (MCC) that sequesters Cdc20, and Mps1 is an essential kinase in the SAC pathway (Musacchio and Hardwick, 2002; Musacchio and Salmon, 2007). Each knockdown drastically decreased the duration of mitotic arrest in kinesin-5 inhibitor, confirming that SAC activity was removed (Figure 5B). Next, we co-knocked down Cdc20 with individual SAC proteins. To avoid competition between siRNA duplexes, HeLa cells were first transfected with Mad2, BubR1, Mps1, or Bub3 siRNA, followed by a second transfection 6 hr later with Cdc20 siRNA. Immunoblots confirmed the efficiency of co-knockdown (Figure 5A). The robust mitotic arrest induced by Cdc20 knockdown was unaffected by co-knockdown of any of the SAC proteins (Figure 5B), confirming that the arrest was SAC independent, as expected from a linear topology of the mitotic arrest pathway (Figure 1A). We then compared the effects of SAC protein knockdown on death induced by kinesin-5 inhibitor with that induced by Cdc20 co-knockdown. Death induced by kinesin-5 inhibitor in HeLa cells was greatly attenuated by knockdown of SAC proteins (Figure 5C), consistent with the view that SAC activity is required for cell killing by conventional spindle-perturbing drugs. Death induced by Cdc20 co-knockdown, in contrast, was unaffected by knockdown of any of the four SAC proteins investigated (Figure 5C). To test whether this result is cell-type dependent, we knocked down Mad2 in the other three lines (Figure 5D). Although mitotic arrest and cell death induced by kinesin-5 inhibitor were sensitive to ablation of Mad2 in all cases, those induced by co-knockdown of Cdc20 were not (Figures 5E and 5F). In each case, death kinetics during mitotic arrest in the absence of Mad2 (Figure 5E) were similar to those in its presence (Figure 2). Similar results were obtained when paclitaxel was used as the antimitotic drug (Figures S2A–S2D). We conclude Cdc20 knockdown is equally effective at killing SAC-competent and SAC-deficient cancer cells, or phrased differently, death induced by knockdown of Cdc20 are SAC independent.

Cdc20 Knockdown Induces Death by MOMP and Non-MOMP Pathways

Antimitotic drugs that work through SAC activation are thought to trigger cell death mainly via the intrinsic, or mitochondrial apoptosis pathway, where the committed step is mitchondrial outer membrane permeabilization (MOMP) (Wang et al., 1999; Park et al., 2004; Tao et al., 2005; Bergstralh and Ting, 2006; Letai, 2008). To confirm this, and to score activation of this pathway in live cells, we generated stable cell lines expressing a previously validated live-cell reporter for MOMP, IMS-RP (Albeck et al., 2008). IMS-RP was created by fusing RFP to the mitochondrial import sequence of Smac (residues 1–59) (Albeck et al., 2008). MOMP during mitotic arrest was evident in HeLa-IMS-RP cells treated with kinesin-5 inhibitor. After many hours of arrest, IMS-RP relocalized abruptly from a punctate, mitochondrial distribution to a smooth, cytosolic distribution. Ten to thirty minutes later (our accuracy was limited by the time-lapse interval), cells initiated vigorous blebbing, followed by complete cessation of movement that we scored as cell death

(Figure S3A, n > 50). When Bcl2, a negative regulator of MOMP, was overexpressed in death-sensitive HeLa-IMS-RP cells, MOMP was prevented as expected. In cells arrested in kinesin-5 inhibitor, IMS-RP remained its punctate mitochondrial distribution, and cells eventually slipped out of arrest with mitochondria intact, and survived until the end of the experiment (Figure S3B, n > 30). These observations confirm that death during mitotic arrest induced by kinesin-5 inhibitor in HeLa occurs by the intrinsic, MOMP-dependent apoptotic pathway. MOMP also did not occur during mitotic arrest in naturally death-resistant A549-IMS-RP cells. Most of these cells slipped, survived, and went on to attempt another round of division with mitochondria intact (Figure S3C, n > 50).

We used the MOMP reporter to address whether Cdc20 knockdown also causes cell death by intrinsic apoptosis. In HeLa-IMS-RP cells knocked down for Cdc20, MOMP during mitotic arrest was unambiguously scored by eye 10–30 min prior to morphological cell death (Figure 6A, n > 50). As an unbiased check on this visual observation, we measured standard deviation of the pixel intensity of the MOMP reporter, and found that it dropped sharply prior to death, as the probe dispersed through the cytoplasm (Figure 6B). In A549-IMS-RP cells knocked down of Cdc20, MOMP was also triggered after extended mitotic arrests (Figure S3D, n > 40).

HeLa cells overexpressing Bcl2 were also efficiently killed by Cdc20 knockdown (Figure 2E). Because MOMP is strongly inhibited in these cells, we wondered if this death, which occurred ~2.5 fold more slowly than in wild-type HeLa, was still correlated with MOMP. By eye, we observed many cases where the reporter appeared to remain punctate as a cell died during mitotic arrest. To quantify this, we defined MOMP-uncorrelated death by failure to detect a sharp decrease in standard deviation of whole-cell IMS-RP pixel intensity 0–1 hr before initiation of gross morphological change leading to death in the phase-contrast channel. More than 80% HeLa overexpressing Bcl2 underwent MOMP-uncorrelated death by this criterion (Figures 6C and 6D, n > 40). The remaining 20% were either MOMP correlated or ambiguous.

Combining these data, when MOMP was allowed, all death events caused by prolonged mitotic arrest, including the unusually long arrest required to kill resistant A549 cells in Cdc20 knockdown, were MOMP correlated. When MOMP was blocked by overexpressing Bcl2 in HeLa, cells died anyway, ~2.5-fold more slowly, but now the death was MOMP uncorrelated and presumably occurred by a different pathway from intrinsic apoptosis.

An Alternative Method for Blocking Mitotic Exit Has Effects Similar to Cdc20 Knockdown

To test whether efficient, SAC-independent induction of death during mitotic arrest was specific for Cdc20 knockdown, or a general consequence of blocking mitotic exit, we expressed human cyclin B1 lacking its destruction box (residues 108–433), fused to EGFP at its C terminus (CT-cyclin-B1-EGFP) (Bentley et al., 2007). This mutant form of cyclin B1 is resistant to APC/C-mediated ubiquitination, and known to cause robust mitotic arrest (Wolf et al., 2006). Immunoblotting confirmed expression of degradation-resistant cyclin B1 and increased level of endogenous cyclin B1 in HeLa cells (Figure S4A). Expression of this mutant cyclin B1 caused efficient mitotic blockade, and efficient cell killing, which was unaffected by RNAi knockdown of SAC proteins (Figures S4B and S4C). We conclude that the precise mechanism by which mitotic exit is blocked is not important for efficient killing of cancer cells.

DISCUSSION

Blocking Mitotic Exit versus Perturbing Spindle Assembly as Cancer Cell Killing Mechanisms

All approved antimitotic drugs, which target microtubule dynamics, and most experimental, spindle-specific drugs, work at least in part by activating the SAC (Jackson et al., 2007). Possible exceptions are Aurora B kinase inhibitors, which inhibit aspects of the SAC as well as damaging the spindle (Hauf et al., 2003). Several authors have hypothesized that reduced SAC activity in some cancer cells, or increased slippage rate, may reduce sensitivity to killing by spindle-perturbing drugs (Taylor and McKeon, 1997; Shin et al., 2003; Tao et al., 2005; Swanton et al., 2007; Gascoigne and Taylor, 2008; Bekier et al., 2009). Our data support this view, and further show that blocking cells in mitosis by a SAC-independent, slippage-resistant mechanism can trigger death more effectively that a SAC-dependent drug. In death-resistant lines, Cdc20 knockdown was much more effective than kinesin-5 inhibition for promoting cell death, whereas in death-sensitive lines the two treatments were similar. Two effects appear to account for this difference: death was induced during mitotic arrest ~2-fold faster in sensitive than resistant lines, and slippage occurred slightly more slowly in sensitive lines. Because induction of death and slippage occur over similar time scales, and they appear to compete to determine cell fate (as proposed by Gascoigne and Taylor, 2008), the net effect is a large difference in total death in response to kinesin-5 inhibitor, but only a 2-fold slowing of death, with all cells eventually dying, in Cdc20 knockdown. We do not know how common the phenotypes of fast slippage and/or slow apoptosis are in actual human tumors, but the fact that we observed them in two of the four solid tumor derived lines tested suggests they may be common. Perhaps this is one reason why spindle-specific drugs have shown only marginal efficacy against solid tumors (Jackson et al., 2007).

The clinically proven drug paclitaxel causes additional postslippage death compared with the kinesin-5 inhibitor we used in some cell lines, especially in A549 cells, despite promoting the same duration of mitotic arrest (Figure 2 and Table 1; Shi et al., 2008). We do not have a clear molecular explanation to account for this difference in death response; based on morphological clues, we speculate it might come from micronucleation, or microtubule stabilization after cells slip. Although execution of the death pathway is postslippage, it requires a critical duration of mitotic arrest; when we deliberately shortened the duration of arrest by knocking down Mad2 in A549 cells, postslippage death in paclitaxel was strongly inhibited (Figure 2I and Figures S2C and S2D). Although paclitaxel is better at promoting postslippage death in some lines, blocking mitotic exit downstream of the SAC was overall far more effective than either drug at promoting death of cells that enter mitosis.

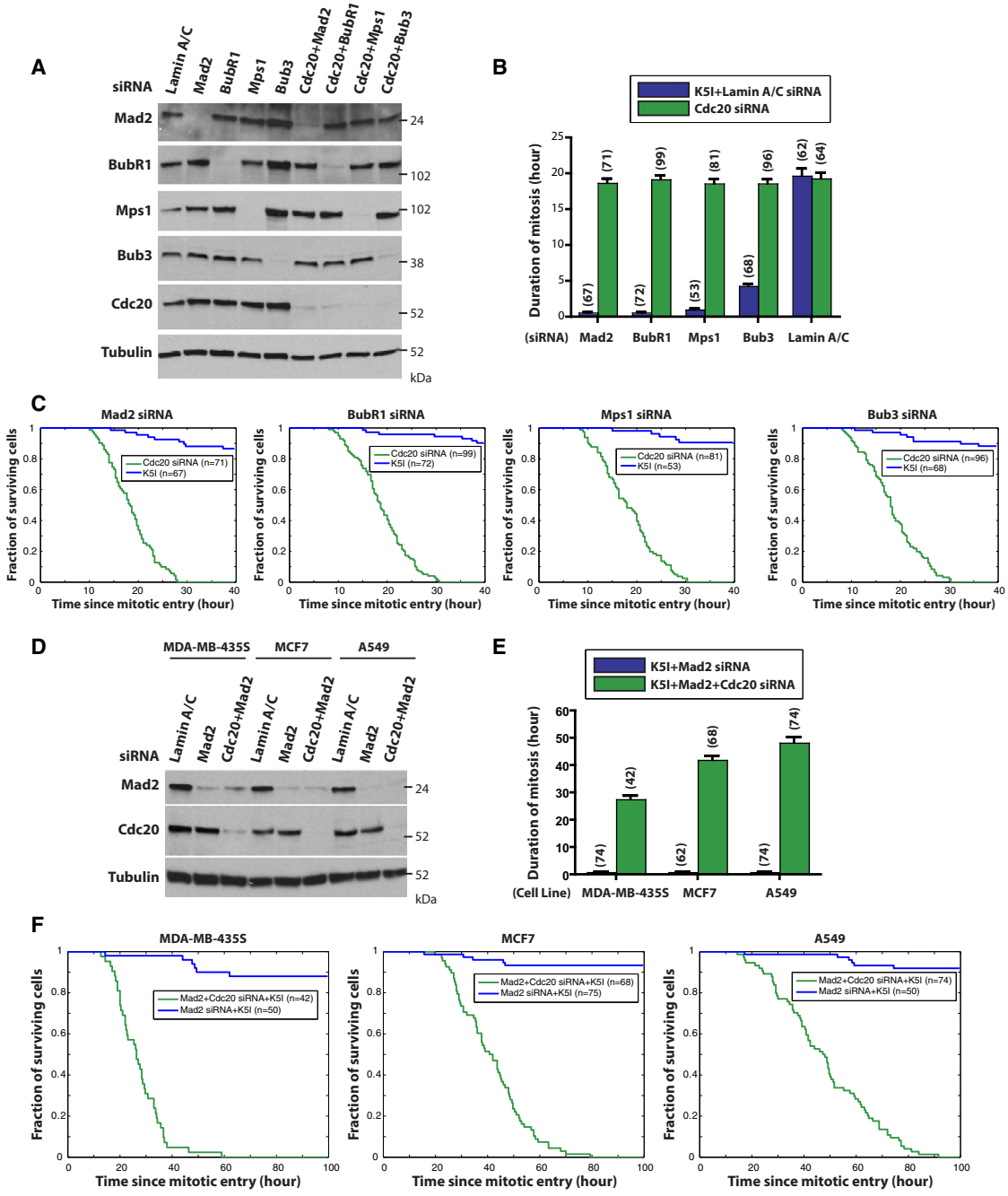

Figure 5. SAC-Independent Mitotic Arrest and Subsequent Death by Knockdown of Cdc20

(A) Knockdown of various SAC proteins and co-knockdown with Cdc20. HeLa cells were transfected with various siRNA duplexes, and cell lysates were immunoblotted as indicated.

(B) Knockdown of SAC proteins abrogates kinesin-5 inhibitor-induced arrest, but has no effect on arrest induced by Cdc20 knockdown. HeLa cells were transfected with indicated SAC protein (or lamin A/C as control) siRNA for 6 hr, followed by a second transfection of Cdc20 siRNA for 6 hr or addition of kinesin-5 inhibitor (with control siRNA). Duration of mitosis was measured by time-lapse microscopy. Numbers in parentheses indicate n. Error bars represent standard deviation (SD).

(C) Knockdown of SAC proteins attenuates kinesin-5 inhibitor-induced death, but has no effect on death induced by co-Cdc20 knockdown. HeLa cells were treated as described in (B), and cumulative survival curves were plotted as a function of time since mitotic entry.

(D) Knockdown of Mad2 and coknockdown with Cdc20. MDA-MB-435S, MCF7 and A549 cells were transfected with siRNA duplexes, and cell lysates were immunoblotted as indicated.

(E) Knockdown of Mad2 abrogates kinesin-5 inhibitor-induced arrest, but has no effect on arrest extended by Cdc20 coknockdown. MDA-MB-435S, MCF7 and A549 cells were treated and analyzed as described in (B). Instead of Cdc20 siRNA transfection alone, kinesin-5 inhibitor was added 12 hr after transfection to improve efficiency of mitotic arrest. Numbers in parentheses indicate n. Error bars represent SD.

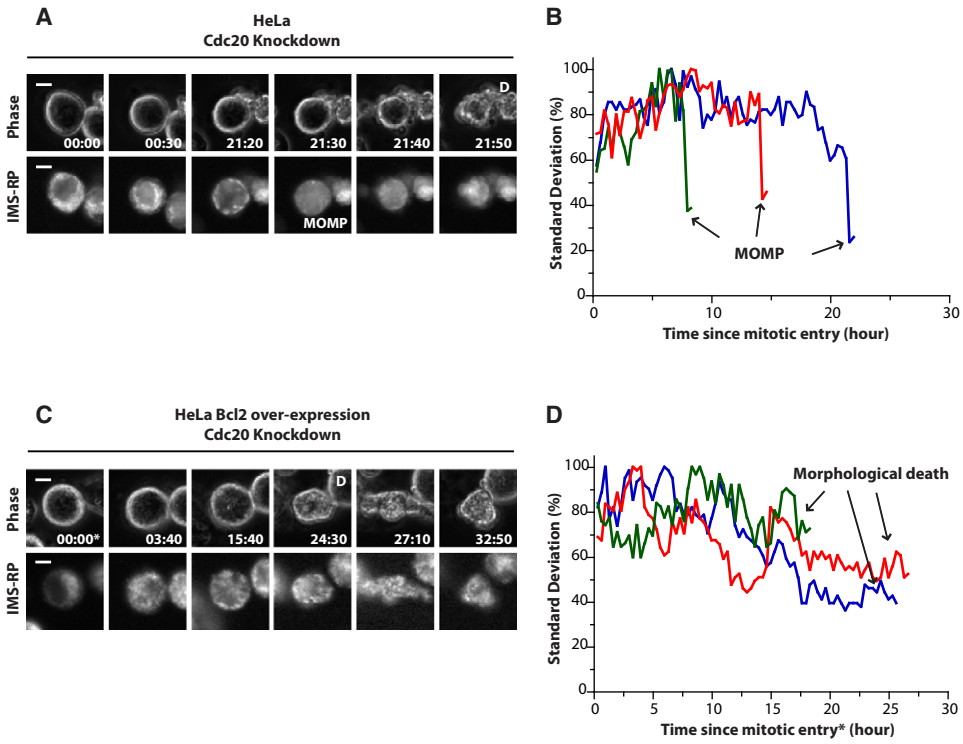

Figure 6. Knockdown of Cdc20 Induces MOMP and Non-MOMP Death

HeLa or HeLa Bcl2-overexpressing cells stably expressing IMS-RP, a red fluorescent protein targeted to the intermembrane space of mitochondria, was used to monitor MOMP. MOMP can be observed from the fluorescence channel, showing a transition from punctate to smooth distribution of IMS-RP. In addition, abrupt decrease in the SD of whole-cell IMS-RP fluorescence intensity (normalized by the highest value) was used to validate MOMP. Time of MOMP is indicated as "MOMP," and time of morphological death is indicated as (D). Numbers indicate elapsed time (hour: minute) relative to mitotic entry. *To avoid extensive photobleaching, cells are prearrested in mitosis by knockdown of Cdc20 before the start of time-lapse imaging (T = 0).

(A) Phase-contrast and IMS-RP time-lapse sequence of a representative HeLa cell transfected with Cdc20 siRNA (shown in blue in B). The scale bar represents 5 μm.

(B) Time course of IMS-RP standard deviation from three representative HeLa cells transfected with Cdc20 siRNA.

(C) Phase-contrast and IMS-RP time-lapse sequence of a representative HeLa Bcl2 overexpresser transfected with Cdc20 siRNA (shown in blue in D). The scale bar represents 5 μm.

(D) Time course of IMS-RP standard deviation from three representative HeLa Bcl2 overexpressers transfected with Cdc20 siRNA.

Cdc20 as a Potential Drug Target

Cdc20 was discovered as an essential gene for cell-cycle progression in budding yeast (Hartwell et al., 1973), and was recently identified in dropout screens for genes that are required for human cancer cell proliferation (Schlabach et al., 2008). Whether Cdc20 is absolutely required for mitotic exit in human cells is still controversial (Li et al., 2007; Wolthuis et al., 2008; Baumgarten et al., 2009). In this study, we showed that siRNA knockdown of Cdc20 causes prolonged mitotic arrest in all lines tested, and it can be rescued by an RNAi-resistant transgene in at least one line. This argues against the existence of APC-independent mitotic exit pathways (Clarke, 2009).

Is Cdc20 is a "druggable target" in the sense that potent, specific small-molecule antagonists could be developed? The most obvious inhibition strategy would be a small molecule that binds to APC/C and competes at the Cdc20 binding site, or vice versa. However, this may not be the only option. MCC participates in complex interactions with various E3s and DUBs (enzymes that add and remove ubiquitin) (Reddy et al., 2007; Stegmeier et al., 2007), and Cdc20 is thought to undergo fast turnover during mitosis in some cells (Nilsson et al., 2008). Thus, it might be possible to remove Cdc20 by antagonizing its translation or deubiquitination. A negative for druggability of Cdc20 is that it must be almost completely inhibited (<5% activity remaining [Wolthuis et al., 2008]) to block mitotic exit, so mitotic arrest by Cdc20 inhibition alone might require a potent inhibitor. However, Cdc20 inhibitors need not be used alone. Combined with a conventional antimitotic drug, Cdc20 inhibitors should suppress slippage, and thus potentiate cell killing.

Other proteins required for mitotic exit could also be considered as targets. Similar effects of Cdc20 knockdown and degradation-resistant cyclin B1 expression suggest that any blockade to mitotic exit will have the same lethal effect on cancer cells. One approach to finding a druggable target in mitotic exit would

(F) Knockdown of Mad2 attenuates kinesin-5 inhibitor-induced death, but has no effect on death induced by Cdc20 coknockdown. MDA-MB-435S, MCF7 and A549 cells were treated and analyzed as described in (C). Mad2 was used as a representative SAC protein, and kinesin-5 inhibitor combined with Cdc20 knockdown was used instead of Cdc20 knockdown alone to improve efficiency of mitotic arrest.

be cell-based screening for mitotic arrest in cells where the SAC has been ablated. SAC ablation would eliminate the large number of tubulin inhibitors that dominate hits from conventional cell-based screens for mitotic arrest (Mayer et al., 1999).

Implication for the Death-Triggering Mechanism during Mitotic Arrest

A major unsolved question for antimitotic drugs is the molecular mechanism by which spindle damage triggers death during mitotic arrest. One long-standing question is the SAC's role in this process (Taylor and McKeon, 1997; Shin et al., 2003; Tao et al., 2005; Swanton et al., 2007; Gascoigne and Taylor, 2008; Bekier et al., 2009). Because mitotic arrest and SAC activation are normally coupled, simply ablating the SAC and showing reduced apoptosis in drugs does not distinguish whether the SAC triggers apoptosis directly, or only indirectly, by promoting arrest (Weaver and Cleveland, 2005). We uncoupled arrest from SAC activation, by using Cdc20 knockdown or degradation-resistant cyclin B1 expression, to promote an SAC-independent mitotic arrest. We showed that death induction were unaffected by co-knockdown of any of four SAC proteins investigated under these conditions (Figure 5C and Figure S4B). This suggests that some general feature of mitotic arrest, not the SAC activity, is the proximal trigger for apoptosis.

With respect to identifying the prodeath (and prosurvival) signal during mitotic arrest, finding that the SAC is not required for death is somewhat disappointing, because the SAC is a discrete pathway involving a small number of proteins, whereas mitotic arrest is a broad change in cell physiology that perturbs essentially every system in the cell. In death-sensitive HeLa cells, the kinetics of cell death during mitotic arrest were the same for Cdc20 knockdown, two different spindle-damaging drugs, and combinations of either drug with Cdc20 knockdown (Figures 2A and 2F). This suggests that the strength of the signal is unaffected by the state of the mitotic spindle, and is thus unlikely to emanate from any microtubule-based system. This signal seems to be slowly cumulative because long durations of arrest are required to trigger death, and to have some memory, because death that depends on long mitotic arrest can occur several hours after slippage. In most of the cells we studied, the signal eventually triggered MOMP, and blocking MOMP by Bcl2 overexpression slowed death, suggesting the signal impinges on the Bcl2-family circuitry that regulates MOMP (Letai, 2008). However, it may act in others ways because Bcl2-overexpressing cells eventually died in mitotic arrest by a non-MOMP pathway, similar to other situations where stressed cells die by alternative programmed death pathways when the canonical apoptosis pathway is blocked (Degterev and Yuan, 2008). There is a large literature on the molecular nature of the signal, suggesting the involvement of Bcl2, Bcl-xL, and caspase-9 phosphorylation, and various kinase signaling pathways including c-Jun N-terminal kinase, ERK, p38 MAP kinase, and AKT (Bacus et al., 2001; Salah-Eldin et al., 2003; Basu and Haldar, 2003; Deacon et al., 2003; Allan and Clarke, 2007; Mhaidat et al., 2007; Kim et al., 2007). However, no clear and general picture has yet emerged, and it remains an area of intensive study. We speculate that this cumulative, death-inducing signal is generated by one or more of the general changes in cell physiology that occur during mitosis, for example in membrane organization, transcription, translation, metabolism, or signaling. Elucidating this signal will be challenging, but knowing its precise nature is not required to harness it for killing cancer cells that enter mitosis, either by SAC activation for current drugs, or by blocking mitotic exit as we propose.

EXPERIMENTAL PROCEDURES

Cell Lines and Drugs

HeLa, MDA-MB-435S, MCF7, A549, and 293 cells were cultured according to ATCC recommendations. HeLa GFP-β-tubulin line was a gift from Paul Chang, and HeLa Bcl2-overexpression line was a gift from Peter Sorger. Reference spindle-perturbing drugs were used at concentrations that are saturating for mitotic arrest (Shi et al., 2008; Orth et al., 2008): EMD534085 (European patent number WO2005063735, provided by Merck Serono) at 1 μM, and paclitaxel (Sigma) at 200 nM.

siRNA

For Cdc20 depletion, Ambion Silencer Select siRNA against Cdc20 (s2748) (duplex 1) was used in all experiments at a final concentration of 50 nM; Dharmacon ON-TARGETplus siRNA duplex against Cdc20 (J-003225-14) was used as an alternative (duplex 2) at 100 nM. To deplete SAC proteins, Dharmacon siGENOME or ON-TARGETplus duplexes against Mad2 (J-003271-13), BubR1 (D-004101-04), Mps1 (D-004105-03), and Bub3 (D-006826-01) were used at 40 nM. Dharmacon Lamin A/C siRNA duplex was used as controls. siRNA transfection was performed using Lipofectamine 2000 (Invitrogen) or HiPerFect (QIAGEN) according to the manufacturer's instructions.

Plasmids and Virus Production

Two extra silent mutations were introduced to mouse Cdc20 cDNA (Open Biosystems) in the area corresponding to human Cdc20 siRNA duplex 1 by polymerase chain reaction (PCR) mutagenesis. The PCR oligos used are: 5′-CGAAATCCGGAATGACTACTATTTGAATCTTGTAGATTGGAGC-3′ and 5′-GCTCCAATCTACAAGATTCAAATAGTAGTCATTCCGGATTTC-3′. Mouse Cdc20 mutant was subcloned into pBabe-puro retroviral expression vector. Retroviral IMS-RP and full-length cyclin-B1-EYFP construct were gifts from Peter Sorger and Jagesh Shah, respectively. Retrovirus was produced in 293 cells and used to infect HeLa or A549 cells to create stable lines as described previously (Zheng and Cantley, 2007). Adenoviruses expressing vector-EGFP, full-length cyclin-B1-EGFP, and CT-cyclin-B1-EGFP were gifts from Randy King and amplified in 293 cells as described elsewhere (Hoyt and King, 2005).

Immunoblot Analysis

Cell lysates in LDS sample buffer (NuPAGE, Invitrogen) were resolved on 12% Tris-HEPES gels (Pierce) and transferred to nitrocellulose membranes. Immunoblotting was performed according to the manufacturers' recommendations with enhanced chemiluminescence (Amersham). Antibodies against Cdc20 and Bub3 were purchased from Abcam; Mad2, BubR1, tubulin, and actin from Sigma; Mps1 from Upstate; cyclin B1 from Lab Vision.

Time-Lapse Imaging

Cells were seeded in glass-bottom plates (MatTek) in CO_2-independent medium (Invitrogen) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. For fluorescent time-lapse imaging cells were seeded in phenol red-free CO_2-independent medium (Invitrogen). Image acquisition was performed using Nikon TE2000 automated inverted microscope with a 20 × objective enclosed in a humidified incubation chamber maintained at 37°C. Images were collected every 10–30 min with a motorized stage. Images were viewed and analyzed with MetaMorph software (Molecular Dynamics).

SUPPLEMENTAL DATA

Supplemental Data include four figures and can be found with this article online at http://www.cell.com/cancer-cell/supplemental/S1535-6108(09)00293-1.

ACKNOWLEDGMENTS

We thank Andrew Murray, Bin Zheng, and the Mitchison lab for helpful discussions; Peter Sorger, Jagesh Shah, Randy King, and Paul Chang for reagents and suggestions; Rebecca Ward for critical reading of the manuscript; and Jennifer Waters and Lara Petrak in Nikon Imaging Center (HMS) for imaging assistance. This work was supported by NCI CA078048. EMD534085 was supplied by Merck Serono.

Received: March 12, 2009
Revised: July 11, 2009
Accepted: August 24, 2009
Published: October 5, 2009

REFERENCES

Albeck, J.G., Burke, J.M., Aldridge, B.B., Zhang, M., Lauffenburger, D.A., and Sorger, P.K. (2008). Quantitative analysis of pathways controlling extrinsic apoptosis in single cells. Mol. Cell *30*, 11–25.

Allan, L.A., and Clarke, P.R. (2007). Phosphorylation of caspase-9 by CDK1/cyclin B1 protects mitotic cells against apoptosis. Mol. Cell *26*, 301–310.

Bacus, S.S., Gudkov, A.V., Lowe, M., Lyass, L., Yung, Y., Komarov, A.P., Keyomarsi, K., Yarden, Y., and Seger, R. (2001). Taxol-induced apoptosis depends on MAP kinase pathways (ERK and p38) and is independent of p53. Oncogene *20*, 147–155.

Baguley, B.C., Marshall, E.S., Whittaker, J.R., Dotchin, M.C., Nixon, J., McCrystal, M.R., Finlay, G.J., Matthews, J.H., Holdaway, K.M., and van Zijl, P. (1995). Resistance mechanisms determining the in vitro sensitivity to paclitaxel of tumour cells cultured from patients with ovarian cancer. Eur. J. Cancer *31A*, 230–237.

Basu, A., and Haldar, S. (2003). Identification of a novel Bcl-xL phosphorylation site regulating the sensitivity of taxol- or 2-methoxyestradiol-induced apoptosis. FEBS Lett. *538*, 41–47.

Baumgarten, A.J., Felthaus, J., and Wasch, R. (2009). Strong inducible knockdown of APC/CCdc20 does not cause mitotic arrest in human somatic cells. Cell Cycle *8*, 643–646.

Bekier, M.E., Fischbach, R., Lee, J., and Taylor, W.R. (2009). Length of mitotic arrest induced by microtubule-stabilizing drugs determines cell death after mitotic exit. Mol. Cancer Ther. *8*, 1646–1654.

Bentley, A.M., Normand, G., Hoyt, J., and King, R.W. (2007). Distinct sequence elements of cyclin B1 promote localization to chromatin, centrosomes, and kinetochores during mitosis. Mol. Biol. Cell *18*, 4847–4858.

Bergstralh, D.T., and Ting, J.P. (2006). Microtubule stabilizing agents: Their molecular signaling consequences and the potential for enhancement by drug combination. Cancer Treat. Rev. *32*, 166–179.

Brito, D.A., and Rieder, C.L. (2006). Mitotic checkpoint slippage in humans occurs via cyclin B destruction in the presence of an active checkpoint. Curr. Biol. *16*, 1194–1200.

Clarke, D.J. (2009). Strong inducible knockdown of Cdc20 does not cause mitotic arrest in human somatic cells: Implications for cancer therapy? Cell Cycle *8*, 515–516.

Deacon, K., Mistry, P., Chernoff, J., Blank, J.L., and Patel, R. (2003). p38 Mitogen-activated protein kinase mediates cell death and p21-activated kinase mediates cell survival during chemotherapeutic drug-induced mitotic arrest. Mol. Biol. Cell *14*, 2071–2087.

Degterev, A., and Yuan, J. (2008). Expansion and evolution of cell death programmes. Nat. Rev. Mol. Cell Biol. *9*, 378–390.

Deng, J., Carlson, N., Takeyama, K., Dal Cin, P., Shipp, M., and Letai, A. (2007). BH3 profiling identifies three distinct classes of apoptotic blocks to predict response to ABT-737 and conventional chemotherapeutic agents. Cancer Cell *12*, 171–185.

Gascoigne, K.E., and Taylor, S.S. (2008). Cancer cells display profound intra- and interline variation following prolonged exposure to antimitotic drugs. Cancer Cell *14*, 111–122.

Hartwell, L.H., Mortimer, R.K., Culotti, J., and Culotti, M. (1973). Genetic Control of the Cell Division Cycle in Yeast: V. Genetic Analysis of cdc Mutants. Genetics *74*, 267–286.

Hauf, S., Cole, R.W., LaTerra, S., Zimmer, C., Schnapp, G., Walter, R., Heckel, A., van Meel, J., Rieder, C.L., and Peters, J.M. (2003). The small molecule Hesperadin reveals a role for Aurora B in correcting kinetochore-microtubule attachment and in maintaining the spindle assembly checkpoint. J. Cell Biol. *161*, 281–294.

Hoyt, J., and King, R.W. (2005). Adenoviral expression of reporter proteins for high-throughput cell-based screening. Methods Mol. Biol. *310*, 187–195.

Jackson, J.R., Patrick, D.R., Dar, M.M., and Huang, P.S. (2007). Targeted anti-mitotic therapies: Can we improve on tubulin agents? Nat. Rev. Cancer *7*, 107–117.

Jordan, M.A., and Wilson, L. (2004). Microtubules as a target for anticancer drugs. Nat. Rev. Cancer *4*, 253–265.

Kim, S.H., Juhnn, Y.S., and Song, Y.S. (2007). Akt involvement in paclitaxel chemoresistance of human ovarian cancer cells. Ann. N Y Acad. Sci. *1095*, 82–89.

Kutuk, O., and Letai, A. (2008). Alteration of the mitochondrial apoptotic pathway is key to acquired paclitaxel resistance and can be reversed by ABT-737. Cancer Res. *68*, 7985–7994.

Letai, A.G. (2008). Diagnosing and exploiting cancer's addiction to blocks in apoptosis. Nat. Rev. Cancer *8*, 121–132.

Li, M., York, J.P., and Zhang, P. (2007). Loss of Cdc20 causes a securin-dependent metaphase arrest in two-cell mouse embryos. Mol. Cell. Biol. *27*, 3481–3488.

Mayer, T.U., Kapoor, T.M., Haggarty, S.J., King, R.W., Schreiber, S.L., and Mitchison, T.J. (1999). Small molecule inhibitor of mitotic spindle bipolarity identified in a phenotype-based screen. Science *286*, 971–974.

Mhaidat, N.M., Zhang, X.D., Jiang, C.C., and Hersey, P. (2007). Docetaxel-induced apoptosis of human melanoma is mediated by activation of c-Jun NH2-terminal kinase and inhibited by the mitogen-activated protein kinase extracellular signal-regulated kinase 1/2 pathway. Clin. Cancer Res. *13*, 1308–1314.

Milross, C.G., Mason, K.A., Hunter, N.R., Chung, W.K., Peters, L.J., and Milas, L. (1996). Relationship of mitotic arrest and apoptosis to antitumor effect of paclitaxel. J. Natl. Cancer Inst. *88*, 1308–1314.

Musacchio, A., and Hardwick, K.G. (2002). The spindle checkpoint: Structural insights into dynamic signalling. Nat. Rev. Mol. Cell Biol. *3*, 731–741.

Musacchio, A., and Salmon, E.D. (2007). The spindle-assembly checkpoint in space and time. Nat. Rev. Mol. Cell Biol. *8*, 379–393.

Nilsson, J., Yekezare, M., Minshull, J., and Pines, J. (2008). The APC/C maintains the spindle assembly checkpoint by targeting Cdc20 for destruction. Nat. Cell Biol. *10*, 1411–1420.

Orth, J.D., Tang, Y., Shi, J., Loy, C.T., Amendt, C., Wilm, C., Zenke, F.T., and Mitchison, T.J. (2008). Quantitative live imaging of cancer and normal cells treated with Kinesin-5 inhibitors indicates significant differences in phenotypic responses and cell fate. Mol. Cancer Ther. *7*, 3480–3489.

Park, S.J., Wu, C.H., Gordon, J.D., Zhong, X., Emami, A., and Safa, A.R. (2004). Taxol induces caspase-10-dependent apoptosis. J. Biol. Chem. *279*, 51057–51067.

Pusztai, L. (2007). Markers predicting clinical benefit in breast cancer from microtubule-targeting agents. Ann. Oncol. *18* (*Suppl 12*), xii15–xii20.

Reddy, S.K., Rape, M., Margansky, W.A., and Kirschner, M.W. (2007). Ubiquitination by the anaphase-promoting complex drives spindle checkpoint inactivation. Nature *446*, 921–925.

Rieder, C.L., and Maiato, H. (2004). Stuck in division or passing through: What happens when cells cannot satisfy the spindle assembly checkpoint. Dev. Cell *7*, 637–651.

Salah-Eldin, A.E., Inoue, S., Tsukamoto, S., Aoi, H., and Tsuda, M. (2003). An association of Bcl-2 phosphorylation and Bax localization with their functions after hyperthermia and paclitaxel treatment. Int. J. Cancer *103*, 53–60.

Schlabach, M.R., Luo, J., Solimini, N.L., Hu, G., Xu, Q., Li, M.Z., Zhao, Z., Smogorzewska, A., Sowa, M.E., Ang, X.L., et al. (2008). Cancer proliferation gene discovery through functional genomics. Science *319*, 620–624.

Shi, J., Orth, J.D., and Mitchison, T. (2008). Cell type variation in responses to antimitotic drugs that target microtubules and kinesin-5. Cancer Res. *68*, 3269–3276.

Shin, H.J., Baek, K.H., Jeon, A.H., Park, M.T., Lee, S.J., Kang, C.M., Lee, H.S., Yoo, S.H., Chung, D.H., Sung, Y.C., et al. (2003). Dual roles of human BubR1, a mitotic checkpoint kinase, in the monitoring of chromosomal instability. Cancer Cell *4*, 483–497.

Sigrist, S., Jacobs, H., Stratmann, R., and Lehner, C.F. (1995). Exit from mitosis is regulated by Drosophila fizzy and the sequential destruction of cyclins A, B and B3. EMBO J. *14*, 4827–4838.

Stegmeier, F., Rape, M., Draviam, V.M., Nalepa, G., Sowa, M.E., Ang, X.L., McDonald, E.R. 3rd, Li, M.Z., Hannon, G.J., Sorger, P.K., Kirschner, M.W., Harper, J.W., and Elledge, S.J. (2007). Anaphase initiation is regulated by antagonistic ubiquitination and deubiquitination activities. Nature *446*, 876–881.

Swanton, C., Marani, M., Pardo, O., Warne, P.H., Kelly, G., Sahai, E., Elustondo, F., Chang, J., Temple, J., Ahmed, A.A., et al. (2007). Regulators of mitotic arrest and ceramide metabolism are determinants of sensitivity to paclitaxel and other chemotherapeutic drugs. Cancer Cell *11*, 498–512.

Symmans, W.F., Volm, M.D., Shapiro, R.L., Perkins, A.B., Kim, A.Y., Demaria, S., Yee, H.T., McMullen, H., Oratz, R., Klein, P., et al. (2000). Paclitaxel-induced apoptosis and mitotic arrest assessed by serial fine-needle aspiration: Implications for early prediction of breast cancer response to neoadjuvant treatment. Clin. Cancer Res. *6*, 4610–4617.

Tan, T.T., Degenhardt, K., Nelson, D.A., Beaudoin, B., Nieves-Neira, W., Bouillet, P., Villunger, A., Adams, J.M., and White, E. (2005). Key roles of BIM-driven apoptosis in epithelial tumors and rational chemotherapy. Cancer Cell *7*, 227–238.

Tao, W., South, V.J., Zhang, Y., Davide, J.P., Farrell, L., Kohl, N.E., Sepp-Lorenzino, L., and Lobell, R.B. (2005). Induction of apoptosis by an inhibitor of the mitotic kinesin KSP requires both activation of the spindle assembly checkpoint and mitotic slippage. Cancer Cell *8*, 49–59.

Taylor, S.S., and McKeon, F. (1997). Kinetochore localization of murine Bub1 is required for normal mitotic timing and checkpoint response to spindle damage. Cell *89*, 727–735.

Wang, L.G., Liu, X.M., Kreis, W., and Budman, D.R. (1999). The effect of antimicrotubule agents on signal transduction pathways of apoptosis: A review. Cancer Chemother. Pharmacol. *44*, 355–361.

Weaver, B.A., and Cleveland, D.W. (2005). Decoding the links between mitosis, cancer, and chemotherapy: The mitotic checkpoint, adaptation, and cell death. Cancer Cell *8*, 7–12.

Wolf, F., Wandke, C., Isenberg, N., and Geley, S. (2006). Dose-dependent effects of stable cyclin B1 on progression through mitosis in human cells. EMBO J. *25*, 2802–2813.

Wolthuis, R., Clay-Farrace, L., van Zon, W., Yekezare, M., Koop, L., Ogink, J., Medema, R., and Pines, J. (2008). Cdc20 and Cks direct the spindle checkpoint-independent destruction of cyclin A. Mol. Cell *30*, 290–302.

Zheng, B., and Cantley, L.C. (2007). Regulation of epithelial tight junction assembly and disassembly by AMP-activated protein kinase. Proc. Natl. Acad. Sci. USA *104*, 819–822.