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## Evidence that mitotic exit is a better cancer therapeutic target than spindle assembly

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### SUMMARY

Current anti-mitotics 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 over-expressed. We conclude targeting Cdc20, or otherwise blocking mitotic exit, may be a better cancer therapeutic strategy than perturbing spindle assembly.

### INTRODUCTION

Anti-mitotic 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 anti-mitotic 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 (aka KSP, Eg5, Kif11), Aurora kinases, and Polo-like kinases (Jackson et al., 2007). In clinical trials to date, these spindle-specific anti-mitotic 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 anti-mitotic 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

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#### SIGNIFICANCE

Drugs targeting spindle proteins, including Kinesin-5, Aurora kinases, and Polo-like kinases, were recently developed in the hope of less neurotoxic anti-mitotic drugs. In clinical trials to date, their efficacy seems no better than taxanes and vincas. We provide evidence that an alternative anti-mitotic 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 towards understanding how mitotic arrest triggers death, and how some cancer cells resist this trigger.

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comparing efficacy for killing cancer cell lines with representative drugs that interfere with spindle assembly.

The net effect of anti-mitotic 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 anti-mitotic drugs, since 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). 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 non-apoptotic outcome following spindle damage and mitotic arrest. First, they may fail to execute apoptosis efficiently due to down-regulation 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 current anti-mitotic 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  $18.8 \pm 7.3$  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 2 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 ( $1.5 \pm 0.9$  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 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 anti-mitotic drugs (Shi et al., 2008); Bcl2 over-expressing 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. Figure 2A–E shows 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. Since 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 pro-death 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, Figure 2A–E 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, > 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, whether Kinesin-5 inhibitor was present or not. 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 to Kinesin-5 inhibitor in cells where we know the origin of death resistance, we used a HeLa line that stably over-expresses Bcl2. Bcl2 antagonizes MOMP, and over-expression of Bcl2 and related family members has been widely implicated in apoptosis resistance in cancer (Letai, 2008). More than 70% of HeLa cells over-expressing 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 Figure 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 over-expressing Bcl2 40.8 hr. Thus, death induction rates during mitotic arrest were ~2.5 fold faster in the most death-sensitive line compared to 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 to >70% in MCF7, A549 and HeLa over-expressing Bcl2) because slippage intervenes to rescue the slower-dying lines, as proposed in the competing pathway model (Gacogne and Taylor, 2008). Finally, in HeLa cells Bcl2 over-expression 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 (Figure 2F–J). In some lines, paclitaxel is more pro-apoptotic 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 over-expressing Bcl2 was intermediate between MCF7 and A549. Overall, while 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 if Cdc20 knockdown perturbs spindle assembly, we imaged microtubules live in HeLa stably expressing GFP- $\beta$ -tubulin (Figure 3). We observed normal bipolar spindles early in the arrest, which gradually became multi-polar 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 Figure 2A–E), 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 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 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 since 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 (Figure 4B and 4D). In this situation, each time course ended when the cell underwent death in mitosis, which occurred on average  $18.8 \pm 7.3$  hr ( $n=98$ ) after mitotic entry in HeLa, and  $43.8 \pm 16.5$  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 (Figure S1). 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<sup>Cdc20</sup> - 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 hrs, so its gradual loss presumably reflects a balance between synthesis and proteolysis (Nilsson et al., 2008). Other mitotic cyclins could potentially contribute to Cdc20 knockdown-mediated mitotic arrest, since depletion of Cdc20 also stabilizes other APC/C<sup>Cdc20</sup> 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 anti-mitotic 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 if 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 if this result is cell type dependent, we knocked down Mad2 in the other three lines (Figure 5D). While 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 (Figure 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 anti-mitotic drug (Figure S2A–D). 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

Anti-mitotic 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 mitochondrial 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 (residue 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 relocated abruptly from a punctate, mitochondrial distribution to a smooth, cytosolic distribution. 10–30 min 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 over-expressed 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 over-expressing Bcl2 were also efficiently killed by Cdc20 knockdown (Figure 2E). Since 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 over-expressing Bcl2 underwent MOMP-uncorrelated death by this criterion (Figure 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 over-expressing 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 if 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). Immunoblots 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 (Figure 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 anti-mitotic 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 than a SAC-dependent drug. In death-resistant lines, Cdc20 knockdown was much more effective than Kinesin-5 inhibition for promoting cell death, while 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 post-slippage death compared to 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 micro-nucleation, or microtubule stabilization after cells slip. Although execution of the death pathway is post-slippage, it requires a critical duration of mitotic arrest; when we deliberately shortened the duration of arrest by knocking down Mad2 in A549 cells, post-slippage death in paclitaxel was strongly inhibited (Figure 2I, S2C and S2D). Although paclitaxel is better at promoting post-slippage 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.

## 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 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 de-ubiquitination. 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 anti-mitotic 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 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 anti-mitotic 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). Since 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 a 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 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 pro-death (and pro-survival) signal during mitotic arrest, finding that the SAC is not required for death is somewhat disappointing, since the SAC is a discrete pathway involving a small number of proteins, while 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 (Figure 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, since long durations of arrest are required to trigger death, and to have some memory, since 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 over-expression slowed death, suggesting the signal impinges on the Bcl2-family circuitry that regulates MOMP (Letai 2008). However, it may act in others ways, since Bcl2 over-expressing 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 (c-JNK), 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- $\beta$ -tubulin line was a gift from Paul Chang, and HeLa Bcl2 over-expression 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  $\mu$ M, and paclitaxel (Sigma) at 200 nM.

### siRNA

To deplete Cdc20, 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 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 PCR mutagenesis. The PCR oligos used are: CGAAATCCGGAATGACTACTATTTGAATCTTGTAGATTGGAGC and GCTCCAATCTACAAGATTCAAATAGTAGTCATTCCGGATTTC. 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 (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 (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 manufacturers' recommendations using 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<sub>2</sub>-independent medium (Invitrogen) supplemented with 10 FBS, 100 U/ml penicillin and 100 µg/ml streptomycin. For fluorescent time-lapse imaging cells were seeded in phenol red-free CO<sub>2</sub>-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 using a motorized stage. Images were viewed and analyzed using MetaMorph software (Molecular Dynamics).

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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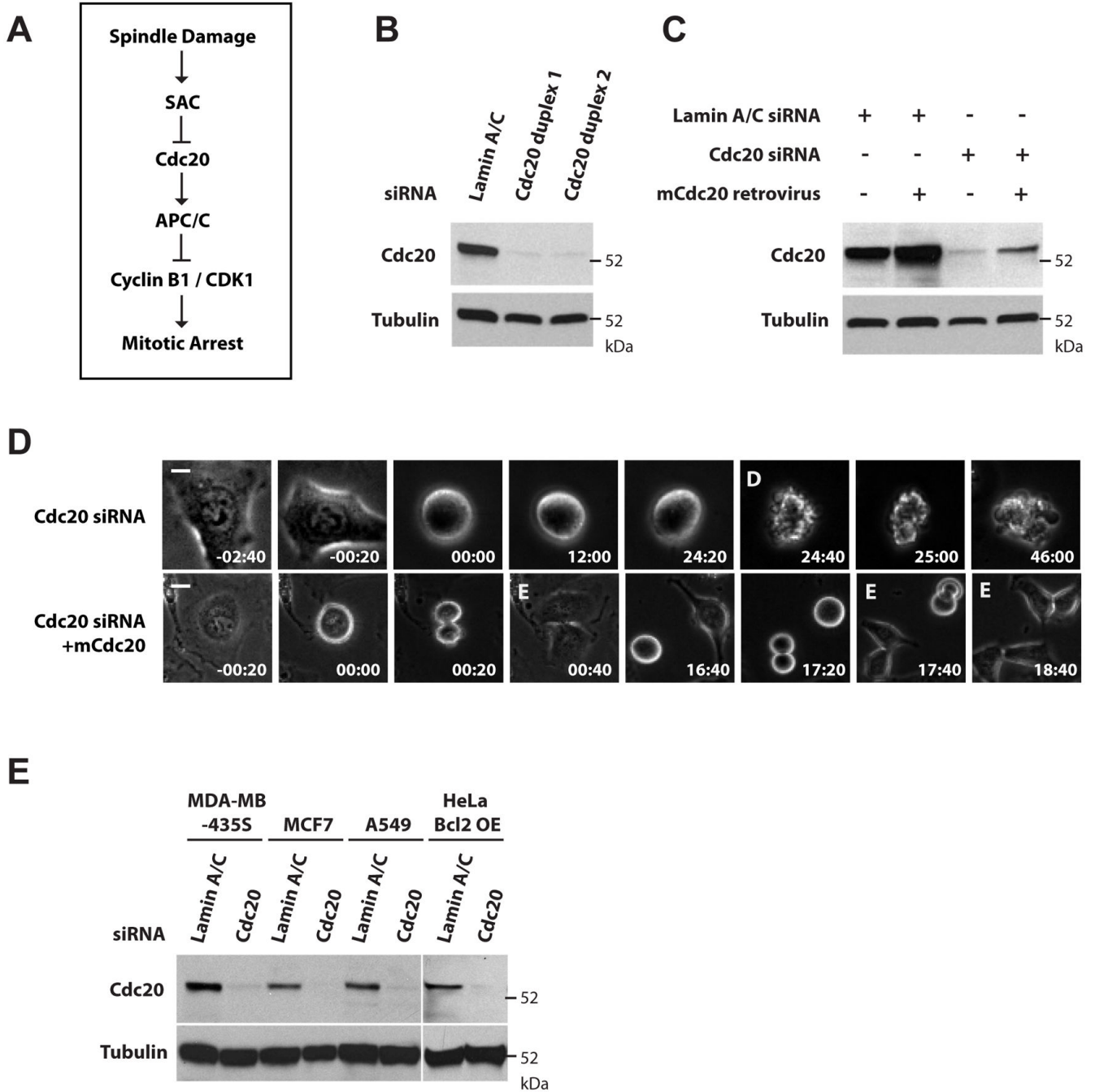
## References

- Albeck JG, Burke JM, Aldridge BB, Zhang M, Lauffenburger DA, Sorger PK. Quantitative analysis of pathways controlling extrinsic apoptosis in single cells. *Mol Cell* 2008;30:11–25. [PubMed: 18406323]
- Allan LA, Clarke PR. Phosphorylation of caspase-9 by CDK1/cyclin B1 protects mitotic cells against apoptosis. *Mol Cell* 2007;26:301–310. [PubMed: 17466630]
- Bacus SS, Gudkov AV, Lowe M, Lyass L, Yung Y, Komarov AP, Keyomarsi K, Yarden Y, Seger R. Taxol-induced apoptosis depends on MAP kinase pathways (ERK and p38) and is independent of p53. *Oncogene* 2001;20:147–155. [PubMed: 11313944]
- Baguley BC, Marshall ES, Whittaker JR, Dotchin MC, Nixon J, McCrystal MR, Finlay GJ, Matthews JH, Holdaway KM, van Zijl P. Resistance mechanisms determining the in vitro sensitivity to paclitaxel of tumour cells cultured from patients with ovarian cancer. *Eur J Cancer* 1995;31A:230–237. [PubMed: 7718330]
- Basu A, Haldar S. Identification of a novel Bcl-xL phosphorylation site regulating the sensitivity of taxol- or 2-methoxyestradiol-induced apoptosis. *FEBS Lett* 2003;538:41–47. [PubMed: 12633850]
- Baumgarten AJ, Felthaus J, Wasch R. Strong inducible knockdown of APC/CCdc20 does not cause mitotic arrest in human somatic cells. *Cell Cycle* 2009;8:643–646. [PubMed: 19197151]
- Bekier ME, Fischbach R, Lee J, Taylor WR. Length of mitotic arrest induced by microtubule-stabilizing drugs determines cell death after mitotic exit. *Mol Cancer Ther* 2009;8:1646–1654. [PubMed: 19509263]
- Bentley AM, Normand G, Hoyt J, King RW. Distinct sequence elements of cyclin B1 promote localization to chromatin, centrosomes, and kinetochores during mitosis. *Mol Biol Cell* 2007;18:4847–4858. [PubMed: 17881737]
- Bergstralh DT, Ting JP. Microtubule stabilizing agents: their molecular signaling consequences and the potential for enhancement by drug combination. *Cancer Treat Rev* 2006;32:166–179. [PubMed: 16527420]

- Brito DA, Rieder CL. Mitotic checkpoint slippage in humans occurs via cyclin B destruction in the presence of an active checkpoint. *Curr Biol* 2006;16:1194–1200. [PubMed: 16782009]
- Broker LE, Kruyt FA, Giaccone G. Cell death independent of caspases: a review. *Clin Cancer Res* 2005;11:3155–3162. [PubMed: 15867207]
- Clarke DJ. Strong inducible knockdown of Cdc20 does not cause mitotic arrest in human somatic cells: implications for cancer therapy? *Cell Cycle* 2009;8:515–516. [PubMed: 19221471]
- Clute P, Pines J. Temporal and spatial control of cyclin B1 destruction in metaphase. *Nat Cell Biol* 1999;1:82–87. [PubMed: 10559878]
- Deacon K, Mistry P, Chernoff J, Blank JL, Patel R. p38 Mitogen-activated protein kinase mediates cell death and p21-activated kinase mediates cell survival during chemotherapeutic drug-induced mitotic arrest. *Mol Biol Cell* 2003;14:2071–2087. [PubMed: 12802076]
- Degterev A, Yuan J. Expansion and evolution of cell death programmes. *Nat Rev Mol Cell Biol* 2008;9:378–390. [PubMed: 18414491]
- Deng J, Carlson N, Takeyama K, Dal Cin P, Shipp M, Letai A. BH3 profiling identifies three distinct classes of apoptotic blocks to predict response to ABT-737 and conventional chemotherapeutic agents. *Cancer Cell* 2007;12:171–185. [PubMed: 17692808]
- Gascoigne KE, Taylor SS. Cancer cells display profound intra- and interline variation following prolonged exposure to antimitotic drugs. *Cancer Cell* 2008;14:111–122. [PubMed: 18656424]
- Hartwell LH, Mortimer RK, Culotti J, Culotti M. Genetic Control of the Cell Division Cycle in Yeast: V. Genetic Analysis of cdc Mutants. *Genetics* 1973;74:267–286. [PubMed: 17248617]
- Hauf S, Cole RW, LaTerra S, Zimmer C, Schnapp G, Walter R, Heckel A, van Meel J, Rieder CL, Peters JM. 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* 2003;161:281–294. [PubMed: 12707311]
- Hoyt J, King RW. Adenoviral expression of reporter proteins for high-throughput cell-based screening. *Methods Mol Biol* 2005;310:187–195. [PubMed: 16353377]
- Jackson JR, Patrick DR, Dar MM, Huang PS. Targeted anti-mitotic therapies: can we improve on tubulin agents? *Nat Rev Cancer* 2007;7:107–117. [PubMed: 17251917]
- Jordan MA, Wilson L. Microtubules as a target for anticancer drugs. *Nat Rev Cancer* 2004;4:253–265. [PubMed: 15057285]
- Kim AH, Bonni A. Thinking within the D box: initial identification of Cdh1-APC substrates in the nervous system. *Mol Cell Neurosci* 2007;34:281–287. [PubMed: 17223572]
- Kim SH, Juhn YS, Song YS. Akt involvement in paclitaxel chemoresistance of human ovarian cancer cells. *Ann N Y Acad Sci* 2007;1095:82–89. [PubMed: 17404021]
- Kutuk O, Letai A. Alteration of the mitochondrial apoptotic pathway is key to acquired paclitaxel resistance and can be reversed by ABT-737. *Cancer Res* 2008;68:7985–7994. [PubMed: 18829556]
- Letai AG. Diagnosing and exploiting cancer's addiction to blocks in apoptosis. *Nat Rev Cancer* 2008;8:121–132. [PubMed: 18202696]
- Li M, York JP, Zhang P. Loss of Cdc20 causes a securin-dependent metaphase arrest in two-cell mouse embryos. *Mol Cell Biol* 2007;27:3481–3488. [PubMed: 17325031]
- Mayer TU, Kapoor TM, Haggarty SJ, King RW, Schreiber SL, Mitchison TJ. Small molecule inhibitor of mitotic spindle bipolarity identified in a phenotype-based screen. *Science* 1999;286:971–974. [PubMed: 10542155]
- Mhaidat NM, Zhang XD, Jiang CC, Hersey P. 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* 2007;13:1308–1314. [PubMed: 17317842]
- Milross CG, Mason KA, Hunter NR, Chung WK, Peters LJ, Milas L. Relationship of mitotic arrest and apoptosis to antitumor effect of paclitaxel. *J Natl Cancer Inst* 1996;88:1308–1314. [PubMed: 8797771]
- Musacchio A, Hardwick KG. The spindle checkpoint: structural insights into dynamic signalling. *Nat Rev Mol Cell Biol* 2002;3:731–741. [PubMed: 12360190]

- Musacchio A, Salmon ED. The spindle-assembly checkpoint in space and time. *Nat Rev Mol Cell Biol* 2007;8:379–393. [PubMed: 17426725]
- Nilsson J, Yekezare M, Minshull J, Pines J. The APC/C maintains the spindle assembly checkpoint by targeting Cdc20 for destruction. *Nat Cell Biol* 2008;10:1411–1420. [PubMed: 18997788]
- Orth JD, Tang Y, Shi J, Loy CT, Amendt C, Wilm C, Zenke FT, Mitchison TJ. 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* 2008;7:3480–3489. [PubMed: 18974392]
- Park SJ, Wu CH, Gordon JD, Zhong X, Emami A, Safa AR. Taxol induces caspase-10-dependent apoptosis. *J Biol Chem* 2004;279:51057–51067. [PubMed: 15452117]
- Peters JM. The anaphase promoting complex/cyclosome: a machine designed to destroy. *Nat Rev Mol Cell Biol* 2006;7:644–656. [PubMed: 16896351]
- Pusztai L. Markers predicting clinical benefit in breast cancer from microtubule-targeting agents. *Ann Oncol* 2007;18(Suppl 12):xii, 15–20.
- Reddy SK, Rape M, Margansky WA, Kirschner MW. Ubiquitination by the anaphase-promoting complex drives spindle checkpoint inactivation. *Nature* 2007;446:921–925. [PubMed: 17443186]
- Rieder CL, Maiato H. Stuck in division or passing through: what happens when cells cannot satisfy the spindle assembly checkpoint. *Dev Cell* 2004;7:637–651. [PubMed: 15525526]
- Salah-Eldin AE, Inoue S, Tsukamoto S, Aoi H, Tsuda M. An association of Bcl-2 phosphorylation and Bax localization with their functions after hyperthermia and paclitaxel treatment. *Int J Cancer* 2003;103:53–60. [PubMed: 12455053]
- Schlabach MR, Luo J, Solimini NL, Hu G, Xu Q, Li MZ, Zhao Z, Smogorzewska A, Sowa ME, Ang XL, Westbrook TF, Liang AC, Chang K, Hackett JA, Harper JW, Hannon GJ, Elledge SJ. Cancer proliferation gene discovery through functional genomics. *Science* 2008;319:620–624. [PubMed: 18239126]
- Shi J, Orth JD, Mitchison T. Cell type variation in responses to antimetabolic drugs that target microtubules and kinesin-5. *Cancer Res* 2008;68:3269–3276. [PubMed: 18451153]
- Shin HJ, Baek KH, Jeon AH, Park MT, Lee SJ, Kang CM, Lee HS, Yoo SH, Chung DH, Sung YC, McKeon F, Lee CW. Dual roles of human BubR1, a mitotic checkpoint kinase, in the monitoring of chromosomal instability. *Cancer Cell* 2003;4:483–497. [PubMed: 14706340]
- Sigrist S, Jacobs H, Stratmann R, Lehner CF. Exit from mitosis is regulated by *Drosophila* fizzy and the sequential destruction of cyclins A, B and B3. *EMBO J* 1995;14:4827–4838. [PubMed: 7588612]
- Stegmeier F, Rape M, Draviam VM, Nalepa G, Sowa ME, Ang XL, McDonald ERr, Li MZ, Hannon GJ, Sorger PK, Kirschner MW, Harper JW, Elledge SJ. Anaphase initiation is regulated by antagonistic ubiquitination and deubiquitination activities. *Nature* 2007;446:876–881. [PubMed: 17443180]
- Swanton C, Marani M, Pardo O, Warne PH, Kelly G, Sahai E, Elustondo F, Chang J, Temple J, Ahmed AA, Brenton JD, Downward J, Nicke B. Regulators of mitotic arrest and ceramide metabolism are determinants of sensitivity to paclitaxel and other chemotherapeutic drugs. *Cancer Cell* 2007;11:498–512. [PubMed: 17560332]
- Symmans WF, Volm MD, Shapiro RL, Perkins AB, Kim AY, Demaria S, Yee HT, McMullen H, Oratz R, Klein P, Formenti SC, Muggia F. 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* 2000;6:4610–4617. [PubMed: 11156210]
- Tan TT, Degenhardt K, Nelson DA, Beaudoin B, Nieves-Neira W, Bouillet P, Villunger A, Adams JM, White E. Key roles of BIM-driven apoptosis in epithelial tumors and rational chemotherapy. *Cancer Cell* 2005;7:227–238. [PubMed: 15766661]
- Tao W, South VJ, Zhang Y, Davide JP, Farrell L, Kohl NE, Sepp-Lorenzino L, Lobell RB. Induction of apoptosis by an inhibitor of the mitotic kinesin KSP requires both activation of the spindle assembly checkpoint and mitotic slippage. *Cancer Cell* 2005;8:49–59. [PubMed: 16023598]
- Taylor SS, McKeon F. Kinetochore localization of murine Bub1 is required for normal mitotic timing and checkpoint response to spindle damage. *Cell* 1997;89:727–735. [PubMed: 9182760]
- Wang LG, Liu XM, Kreis W, Budman DR. The effect of antimicrotubule agents on signal transduction pathways of apoptosis: a review. *Cancer Chemother Pharmacol* 1999;44:355–361. [PubMed: 10501907]

- Weaver BA, Cleveland DW. Decoding the links between mitosis, cancer, and chemotherapy: The mitotic checkpoint, adaptation, and cell death. *Cancer Cell* 2005;8:7–12. [PubMed: 16023594]
- Wolf F, Wandke C, Isenberg N, Geley S. Dose-dependent effects of stable cyclin B1 on progression through mitosis in human cells. *EMBO J* 2006;25:2802–2813. [PubMed: 16724106]
- Wolthuis R, Clay-Farrace L, van Zon W, Yekezare M, Koop L, Ogink J, Medema R, Pines J. Cdc20 and Cks direct the spindle checkpoint-independent destruction of cyclin A. *Mol Cell* 2008;30:290–302. [PubMed: 18471975]
- Zheng B, Cantley LC. Regulation of epithelial tight junction assembly and disassembly by AMP-activated protein kinase. *Proc Natl Acad Sci U S A* 2007;104:819–822. [PubMed: 17204563]



**Figure 1. Cdc20 as a potential anti-mitotic drug candidate**

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

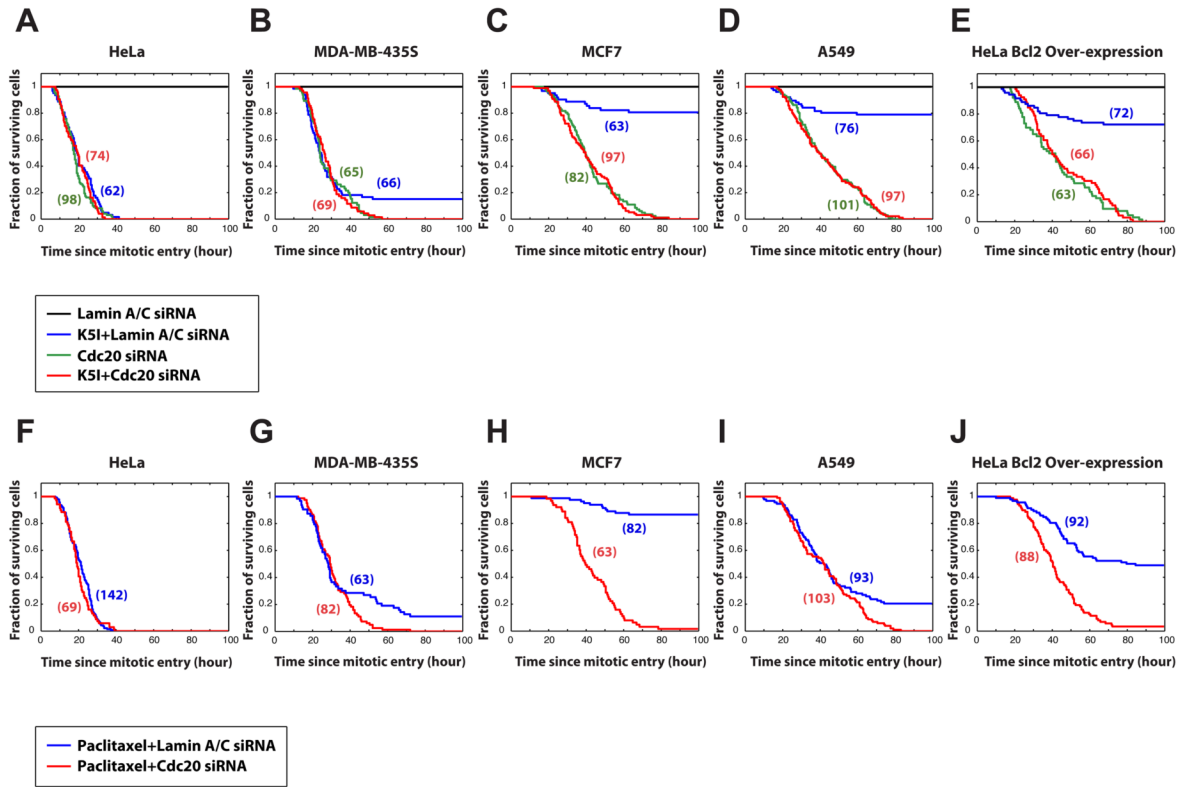
(B) siRNA knockdown of Cdc20 in HeLa cells as analyzed by immunoblotting for Cdc20 and tubulin.

(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

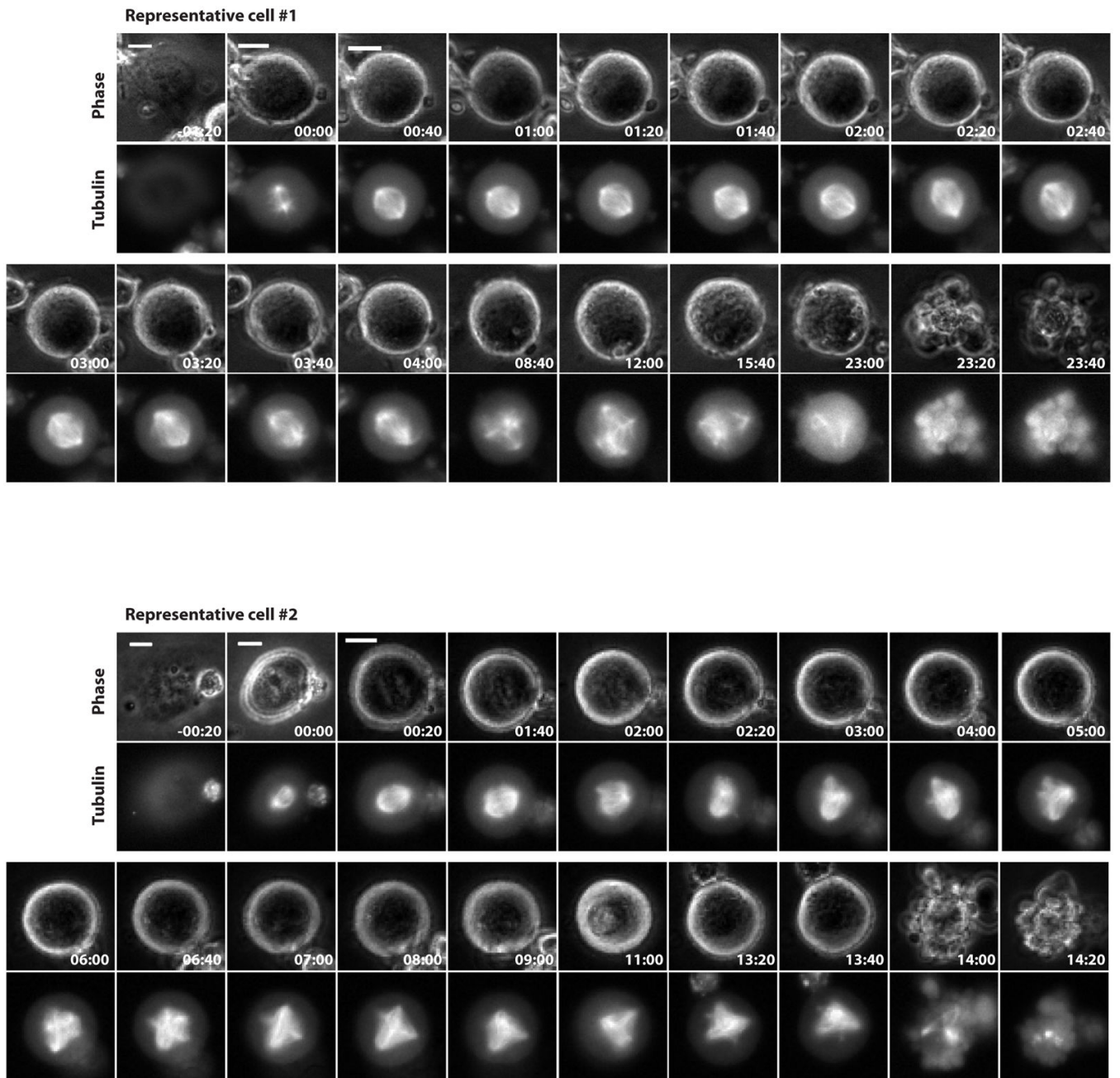
entry. Time of death (to the nearest frame) is indicated as D, and mitotic exit is indicated as E.  
Scale bar: 5  $\mu$ m.

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



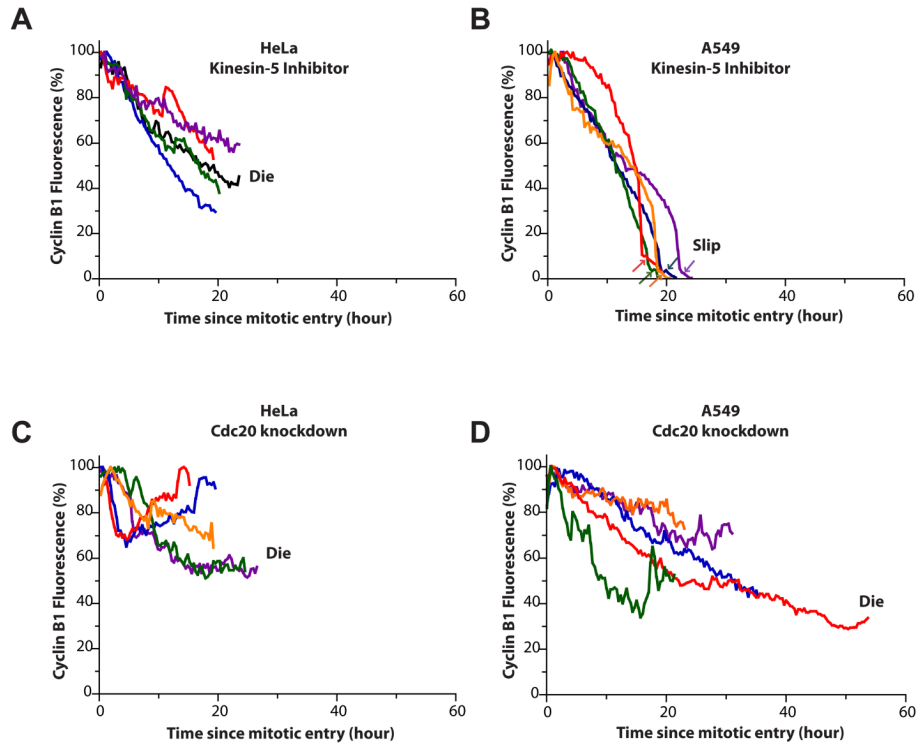
**Figure 2. Knockdown of Cdc20 efficiently kills slippage-prone and apoptosis-resistant cancer cells**  
 Cumulative survival curves for indicated treatments in various cancer cell lines: HeLa (A and F), MDA-MB-435S (B and G), MCF7 (C and H), A549 (D and I), and HeLa cells stably expressing Bcl2 (E and 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.





**Figure 3. Knockdown of Cdc20 induces spindle damage over time**

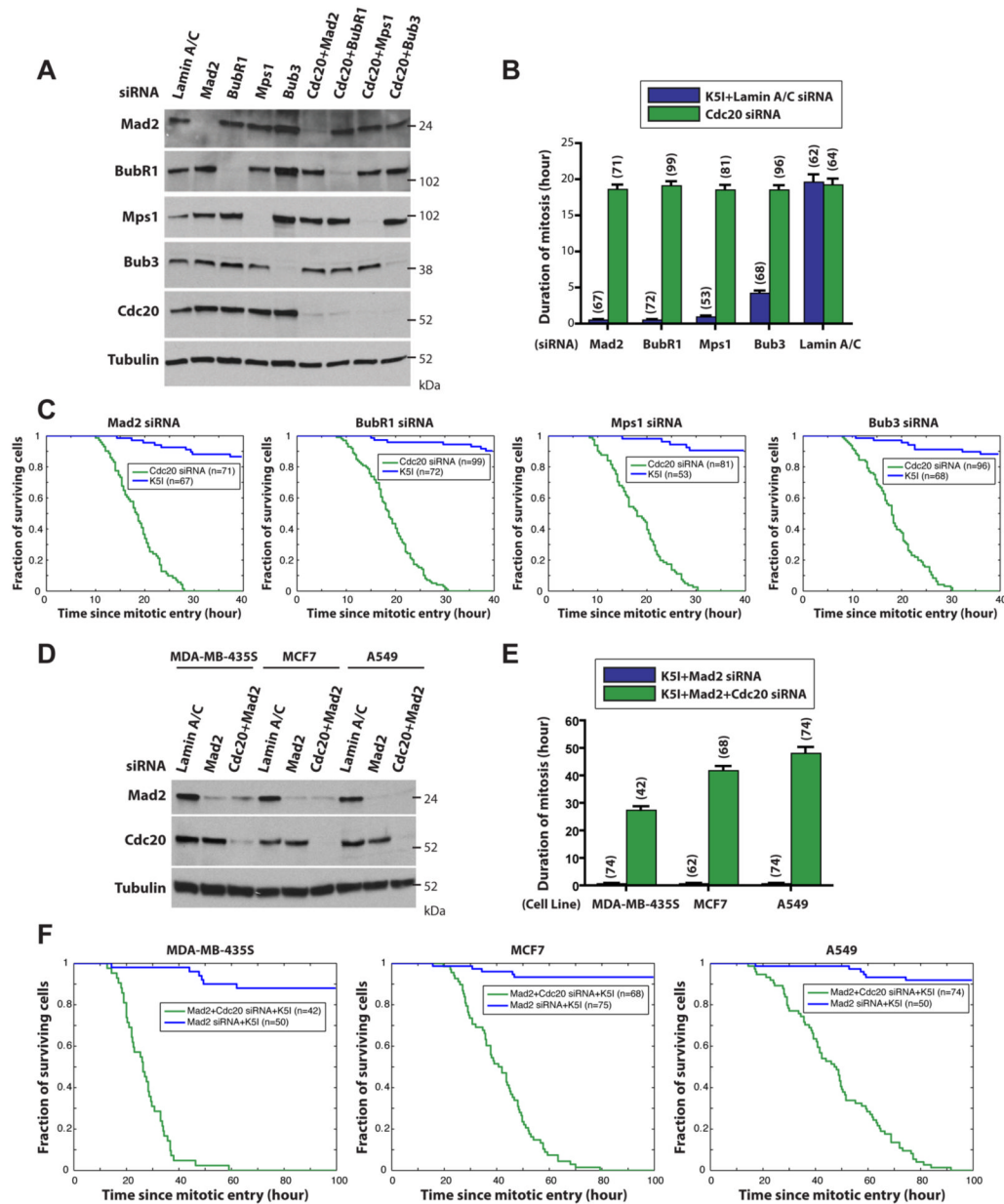
Time evolution of mitotic spindles from 2 representative HeLa-GFP- $\beta$ -tubulin cells. Cells were transfected with Cdc20 siRNA and monitored by time-lapse microscopy. Numbers indicate elapsed time (hour: minute) relative to mitotic entry. Scale bar: 5  $\mu$ m.



**Figure 4. Knockdown of Cdc20 slows cyclin B1 proteolysis**

Changes of cyclin B1 level over time from 5 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.

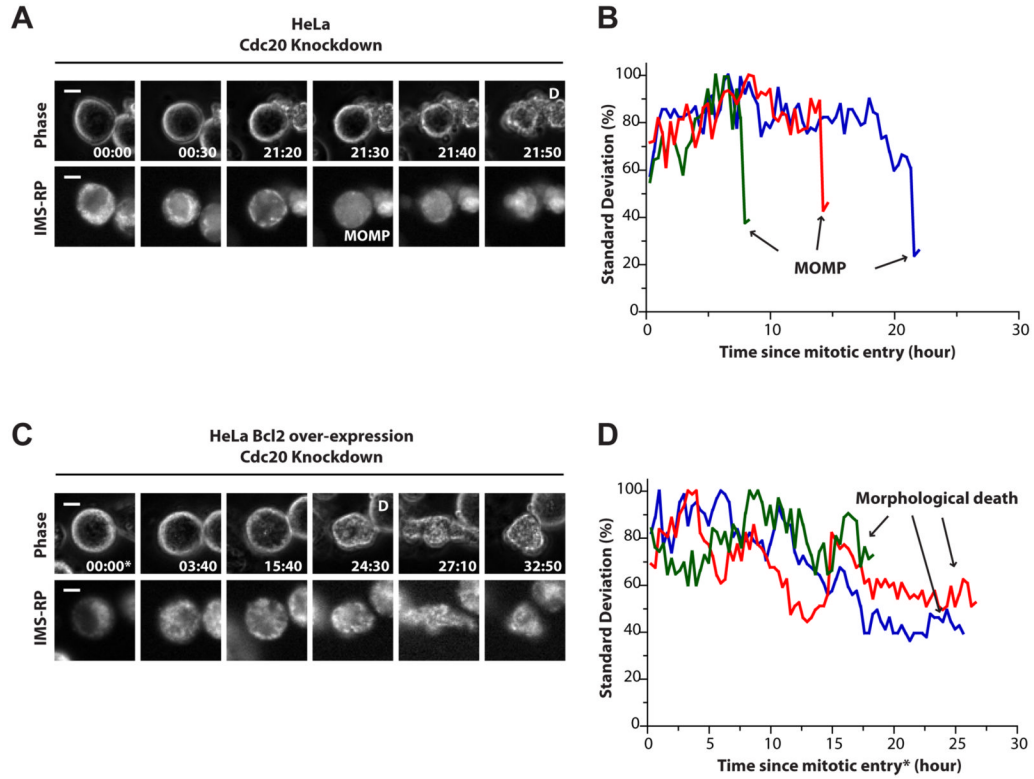
- (A) HeLa cells died in mitosis under Kinesin-5 inhibitor.
- (B) A549 cells slipped out of mitotic arrest induced by Kinesin-5 inhibitor.
- (C) HeLa cells died in mitosis when treated with Cdc20 siRNA.
- (D) A549 cells died in mitosis when treated with Cdc20 siRNA.



**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 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 co-knockdown 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 co-knockdown. 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.

(F) Knockdown of Mad2 attenuates Kinesin-5 inhibitor-induced death, but has no effect on death induced by Cdc20 co-knockdown. 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.



**Figure 6. Knockdown of Cdc20 induces MOMP and non-MOMP death**

HeLa or HeLa Bcl2 over-expressing cells stably expressing IMS-RP, a red fluorescent protein targeted to the inter-membrane 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 photo-bleaching, cells are pre-arrested 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)). Scale bar: 5  $\mu$ m.

(B) Time course of IMS-RP standard deviation from 3 representative HeLa cells transfected with Cdc20 siRNA.

(C) Phase-contrast and IMS-RP time-lapse sequence of a representative HeLa Bcl2 over-expresser transfected with Cdc20 siRNA (shown in blue in (D)). Scale bar: 5  $\mu$ m.

(D) Time course of IMS-RP standard deviation from 3 representative HeLa Bcl2 over-expressers transfected with Cdc20 siRNA.

**Table 1** Duration of mitosis and percentage of cell death in response to Kinesin-5 inhibitor or paclitaxel incubated with Lamin A/C siRNA

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

<sup>a</sup>Percentage of total cell death

<sup>b</sup>Percentage of death in mitosis

<sup>c</sup>Percentage of death after mitotic exit

<sup>d</sup>HeLa cells over-expressing Bcl2