# Caspase activity is not required for the mitotic checkpoint or mitotic slippage in human cells

Kyunghee Lee[a], Alison E. Kenny[a], and Conly L. Rieder[a,b]
[a]Division of Translational Medicine, Biggs Laboratory, Wadsworth Center, New York State Department of Health, Albany, NY 12201–0509; [b]Department of Biomedical Sciences, School of Public Health, State University of New York, Albany, NY 12222

**ABSTRACT** **Biochemical studies suggest that caspase activity is required for a functional mitotic checkpoint (MC) and mitotic slippage. To test this directly, we followed nontransformed human telomerase immortalized human retinal pigment epithelia (RPE-1) cells through mitosis after inhibiting or depleting selected caspases. We found that inhibiting caspases individually, in combination, or in toto did not affect the duration or fidelity of mitosis in otherwise untreated cells. When satisfaction of the MC was prevented with 500 nM nocodazole or 2.5 μM dimethylenastron (an Eg5 inhibitor), 92–100% of RPE-1 cells slipped from mitosis in the presence of pan-caspase inhibitors or after simultaneously depleting caspase-3 and -9, and they did so with the same kinetics (~21–22 h) as after treatment with nocodazole or Eg5 inhibitors alone. Surprisingly, inhibiting or depleting caspase-9 alone doubled the number of nocodazole-treated, but not Eg5-inhibited, cells that died in mitosis. In addition, inhibiting or depleting caspase-9 and -3 together accelerated the rate of slippage ~40% (to ~13–15 h). Finally, nocodazole-treated cells that recently slipped through mitosis in the presence or absence of pan-caspase inhibitors contained numerous BubR1 foci in their nuclei. From these data, we conclude that caspase activity is not required for a functional MC or for mitotic slippage.**

**Monitoring Editor**
Fred Chang
Columbia University

Received: Mar 18, 2011
Revised: May 10, 2011
Accepted: May 17, 2011

## INTRODUCTION

Programmed cell death in humans is mediated by two independent extrinsic and intrinsic pathways, both of which use "initiator" and "executioner" caspases to kill the cell. The former extrinsic pathway is triggered by death receptors that activate the initiator caspase-8 to start a cascade involving "executioner" caspase-3 and/or -7. The latter intrinsic pathway is triggered by nonreceptor stimuli, including cytotoxic stress and DNA damage. The primary initiator of this pathway is caspase-9, while caspase-3 and -7 are the major executioners (Fuentes-Prior and Salvesen, 2004; Pop and Salvesen, 2009). Intrinsic pathways are characterized by permeabilization of the outer mitochondrial membrane and release of cytochrome *c*, and are regulated by pro- and antiapoptotic proteins (Eckelman *et al.*, 2006; Brenner and Mak, 2009). The caspase cascades induced by apoptotic stimuli are, however, flexible; when challenged with inducers of the intrinsic pathway, mouse cells lacking caspase-9 or -3 die from the compensatory activation of caspase-2, -6, and -7 (Zheng *et al.*, 2000).

Spindle poisons like nocodazole or Eg5 kinesin motor inhibitors significantly prolong mitosis by preventing satisfaction of the mitotic checkpoint (MC). Under this condition, cells either die in mitosis via apoptosis or slip into the next G1 as 4N cells (reviewed in Rieder and Maiato, 2004), which, depending on their p53 status, may continue to cycle (Lanni and Jacks, 1998). Whether a cell dies before slipping appears to be dictated by pro- and antiapoptotic signals (Gascoigne and Taylor, 2008; Shi *et al.*, 2008), some of which are regulated by the cyclin B/cyclin-dependent kinase 1 (CDK1) (Allan and Clarke, 2007; Matthess *et al.*, 2010; Terrano *et al.*, 2010). For example, during mitosis in nocodazole-treated HeLa and U2OS cells, the antiapoptotic protein Mcl-1 is phosphorylated by cyclin B/CDK1, which induces its progressive destruction via the Anaphase Promoting Complex (APC)/proteasome pathway (Harley *et al.*, 2010).

These and other studies have fostered the idea that death occurring in spindle poisons is governed by a temporal mechanism that

This article was published online ahead of print in MBoC in Press (http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E11-03-0228) on May 25, 2011.

Address correspondence to: Conly L. Rieder (conlyrieder@hotmail.com).

Abbreviations used: APC, Anaphase Promoting Complex; CDK1, cyclin-dependent kinase 1; DME, dimethylenastron; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; FBS, fetal bovine serum; FMK, Z-VAD(OMe)-FMK; HME, human mammary epithelia; MC, mitotic checkpoint; PARP1, poly(ADP-ribose)polymerase 1; PBS, phosphate-buffered saline; QVD, Q-VD-OPH; RPE-1, telomerase immortalized human retinal pigment epithelia; XIAP, X-linked inhibitor of apoptosis protein.

modulates the balance between pro- and antiapoptotic factors, killing cells in mitosis if the delay extends past a critical point. In addition to influencing the balance between pro- and antiapoptotic factors, cyclin B/CDK1 activity is also reported to restrain certain apoptotic routes in nocodazole-treated HeLa and U2OS cells by directly inhibiting caspase-2 (Andersen *et al.*, 2009) and -8 (Matthess *et al.*, 2010). Indeed, work on cells treated with Eg5 inhibitors (Tao *et al.*, 2007; Vijapukar *et al.*, 2007; Kozielski *et al.*, 2008), Taxol (Rensen *et al.*, 2009; Xu *et al.*, 2009; Peterson *et al.*, 2010), or nocodazole (Allan and Clarke, 2007; Shi *et al.*, 2008) reveals that apoptosis during mitosis occurs primarily via the intrinsic pathway involving caspase-9 and -3.

Biochemical studies of HeLa and other cancer cells harvested after a prolonged drug-induced block in mitosis suggest that in addition to its role in programmed cell death, caspase activity is also required for normal MC function (Swe and Sit, 2000; Hsu *et al.*, 2006; Hashimoto *et al.*, 2008) as well as mitotic slippage. The latter is postulated to result from a requirement that BubR1 be destroyed in a caspase-dependent manner during slippage (Baek *et al.*, 2005; Kim *et al.*, 2005, 2008). The idea that caspase activity is required for slippage is supported by indirect (e.g., Kim *et al.*, 2005) and direct (Gascoigne and Taylor, 2008) data that reveal pan-caspase inhibitors significantly prolong the duration of a mitotic block induced by spindle poisons for many cancer lines. This is also true for at least one nontransformed cell line (human mammary epithelia [HME]), both for cells that die in mitosis and also for those that ultimately slip into the next G1 (Gascoigne and Taylor, 2008).

Other interpretations of these data are, however, equally likely. For example, pan-caspase inhibitors may prolong mitosis in spindle poisons not because caspase activity is required for slippage, but because pan-caspase inhibitors delay death in the sizable portion of the population that normally dies in mitosis being incapable of slipping (Brito and Rieder, 2009). Similarly, since human Bub1 is cleaved during apoptosis (Perera and Freire, 2005), the destruction of BubR1 during a prolonged mitotic arrest may simply reflect a progressive increase in the number of cells dying in mitosis, and not slippage. To examine these issues directly and in more detail, we used live-cell imaging to determine the effect of inhibiting or knocking down caspases, individually or in combination, on mitosis in nontransformed human telomerase immortalized human retinal pigment epithelia (RPE-1) cells. Unlike transformed cell lines, the RPE-1 line is karyotypically and genetically stable and thus retains a highly homogenous genetic population from passage to passage (Kim *et al.*, 2002). As a result, even after long-term culture, RPE-1 retain the capacity to undergo terminal differentiation in vitro (Rambhatla *et al.*, 2002).

## RESULTS

For most of our studies, we followed fields of untreated or experimentally treated RPE-1 cells at 37°C for up to 48 h, at one frame every 1 (Table 1) or 10 (Supplemental Table S1) min, using low-magnification, phase-contrast light microscopy. We then analyzed these records to determine the fate of individual cells and the duration of mitosis, which we defined as the period between the first signs of cell rounding to the first signs of cytokinesis or the membrane blebbing associated with telophase (Supplemental Figure S1).

For our initial studies, we used two pan-caspase inhibitors: the popular Z-VAD(OMe)-FMK (FMK), as well as the newer, more potent but less toxic Q-VD-OPH (QVD). Others have concluded that any experimental design to determine caspase (C) dependency should include both of these pan-caspase inhibitors (Caserta *et al.*, 2003; Chauvier *et al.*, 2007). We used QVD, which is equally effective in preventing apoptosis mediated by the three major pathways: C9/3, C8/10, and C12, at 50 µM. This is 10 times the concentration required to completely protect WEHI cells from actinomycin-induced apoptosis (Caserta *et al.*, 2003), and one that completely prevents apoptosis during mitosis in nocodazole-treated RPE-1 cells (Brito and Rieder, 2009). We used FMK at 100 µM, a concentration that has been shown to block almost all PARP cleavage and apoptosis in nocodazole-treated HeLa cells (Shi *et al.*, 2008), and that also completely inhibits apoptosis in nocodazole-treated RPE-1 cells (see below). We also augmented our studies, where necessary, by inhibiting or knocking down C3, C8, and C9 individually or in combination (Figure 1, Table 1).

### Caspase activity is not required for a normal mitosis

When RPE-1 cultures were treated with pan-caspase inhibitors (QVD or FMK) the cells entered and completed mitosis on average in 21–22 min, the same as nontreated and inactive analogue-treated controls. Inhibiting or knocking down C9, C8, and/or C3, which are the primary initiator and executioner caspases for apoptosis during

| Lane | Caspase inhibitor/RNAi | Duration of mitosis (h)[a] | n |
|---|---|---|---|
| 1 | Control (no treatment) | 20.7 ± 4.8 | 370 |
| 2 | Q-VD-OPh (50 µM) | 21.9 ± 9.0 | 138 |
| 3 | Control (analogue) | 21.2 ± 5.7 | 356 |
| 4 | Z-VAD(OMe)-FMK (50 µM) | 22.5 ± 8.0 | 79 |
| 5 | RNAi scrambled control | 21.9 ± 5.0 | 124 |
| 6 | C8-I (50 µM) | 21.1 ± 5.0 | 92 |
| 7 | C8 RNAi | 22.6 ± 5.7 | 80 |
| 8 | C9-I (50 µM) | 21 ± 8.0 | 334 |
| 9 | C9 RNAi | 21.3 ± 5.3 | 323 |
| 10 | C3-I (50 µM) | 21 ± 7.7 | 154 |
| 11 | C3 RNAi | 21.9 ± 5.8 | 250 |
| 12 | C3 + C9 RNAi | 22.4 ± 7.3 | 234 |

[a]First signs of cell rounding to the first signs of cytokinesis.
C8-I = C8 inhibitor; C9-I = C9 inhibitor; C3-I = C3 inhibitor.

**TABLE 1:** Duration of mitosis at 37°C in RPE-1 cells after inhibiting or depleting caspases individually or in combination.

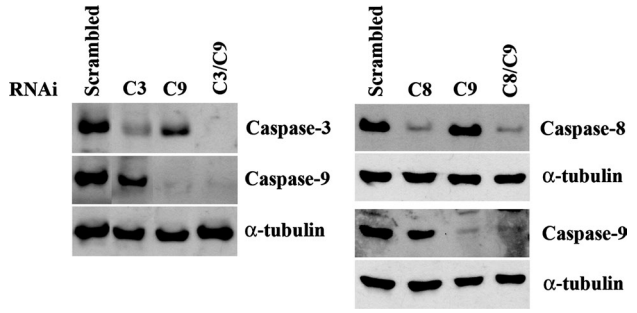


FIGURE 1: RNAi treatment significantly depletes C3 and/or -9 (left), or C8 and/or -9 (right). RPE-1 cultures were transfected with RNAi duplexes as indicated, and their lysates immunoblotted 48 h later for caspases. α-Tubulin was used as a loading control.

mitosis, also had no effect on the duration of mitosis in otherwise untreated cultures (Table 1).

To determine whether caspase activity is required for a normal mitosis, we treated RPE-1 cultures with QVD or a C9 inhibitor. We fixed the cultures after 16 h and compared the ratio of prometaphase, metaphase, and anaphase cells with those of untreated control cultures. As expected from our timing data, we found no significant differences in the ratios or the mitotic stages or in the mitotic indices (Figure 2, A and B), confirming that the duration of spindle assembly was the same in both. We also found no lagging chromosomes in any of the control or experimentally treated anaphase cells (Figure 2C). We conclude from these studies that caspase activity is not required in RPE-1 cells for a normal mitosis.

## Caspase activity is not required for a functional MC or for mitotic slippage

To determine whether caspase activity is required for a functional MC and/or mitotic slippage, we followed fields of RPE-1 cells for up to 48 h after treating them with a concentration of nocodazole (500 nM) or an Eg5 inhibitor (2.5 μM dimethylenastron [DME]; Gartner *et al.*, 2005) known to prevent MC satisfaction in RPE-1 (Brito *et al.*, 2008). Analyses of these records revealed 76% of the cells treated with nocodazole successfully slipped through mitosis on average after ~21 h, while the remainder (24%) died in mitosis after ~21 h (Figure 3A and Table S1, lane 1). A similar result was seen with the Eg5 inhibitor, where 83% of the cells slipped from mitosis into G1 after an average of ~22 h, while 17% died in mitosis after ~20 h (Figure 4A and Table S1, lane 11). Thus, as previously reported (Brito and Rieder, 2006; Gascoigne and Taylor, 2008; Shi *et al.*, 2008), the majority of nontransformed RPE-1 cells survive to slip out of mitosis when the MC cannot be satisfied.

Next we repeated this study in the presence of QVD or FMK. Under these conditions, both pan-caspase inhibitors eliminated all of the nocodazole-induced death in mitosis, and they either eliminated (QVD) or nearly eliminated (FMK) death in mitosis induced by DME (Figures 3, B and C, and 4, B and C, and Table S1, lanes 2–3 and 12–13). Thus, we are using our pan-caspase inhibitors at an effective concentration and for RPE-1 when the MC cannot be satisfied all death in mitosis is due to apoptosis. Importantly, we also found no significant difference ($p \geq 0.05$) in the duration of mitosis in nocodazole- or Eg5-inhibited cells that were also exposed to FMK or QVD: in either pan-caspase inhibitor the cells averaged 20–23 h in mitosis before slipping and 96–100% survived to slip (Figures 3, A–C, and 4, A–C, and Table S1, lanes 1–3 and 11–13). We conclude from these data that caspase activity is not required in RPE-1 for a functional MC, and that inhibiting caspase activity in toto with pan-caspase inhibitors does not prolong mitosis in RPE-1 when the MC cannot be satisfied.

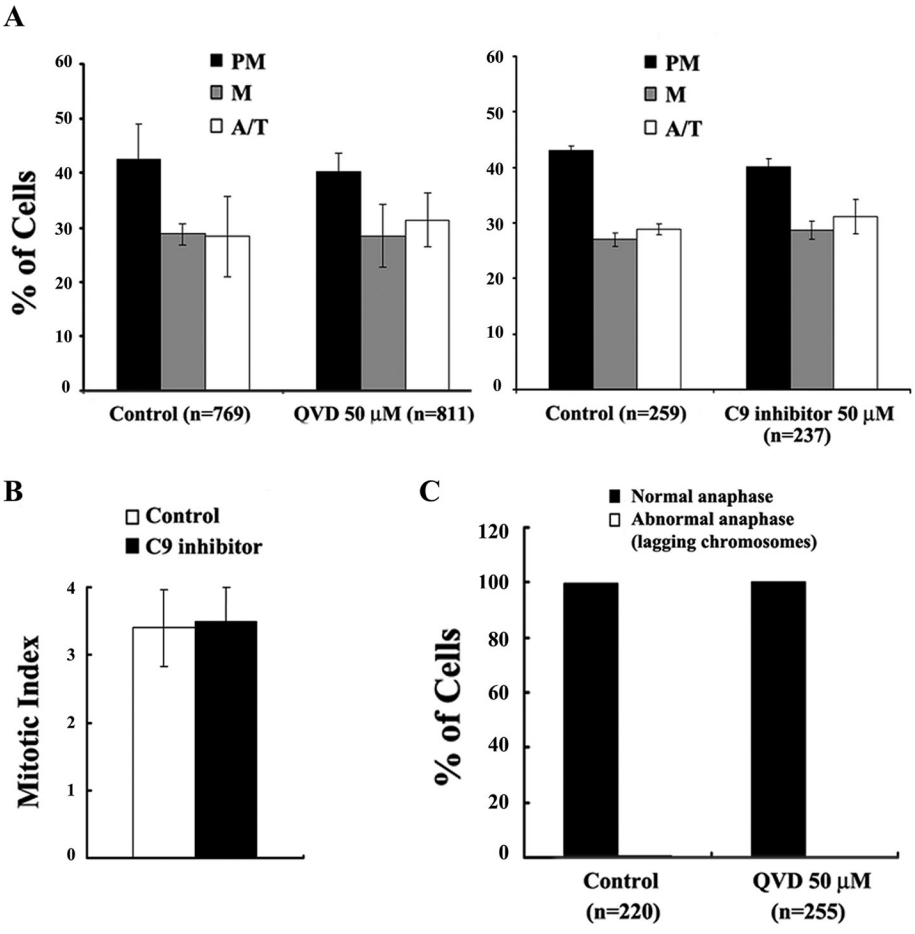


FIGURE 2: Inhibiting caspase activity in growing RPE-1 cultures with a pan-caspase inhibitor (QVD) or a C9 inhibitor does not lead to defects in mitosis. Under both conditions, there is no change in the ratios of the mitotic stages (A) or in the mitotic index (B; percent of cells in mitosis), and it does not lead to lagging chromosomes in anaphase cells (C). n = number of mitotic cells in each data set; PM = prometaphase; M = metaphase; A/T = anaphase/telophase.

## Inhibiting/deleting just C9 significantly enhances death during mitosis in cells treated with nocodazole but not Eg5 inhibitors

As noted in the *Introduction*, apoptotic cell death in the presence of spindle poisons during mitosis appears to be mediated primarily by the C9/C3 pathway in those lines where it has been examined. To confirm this for RPE-1, we inhibited or knocked down C9 and C3, individually or in combination. As reported for transformed cell lines, significantly more RPE-1 cells survived to slip through mitosis when we simultaneously depleted ($p \leq 0.05$) or inhibited ($p \leq 0.05$)

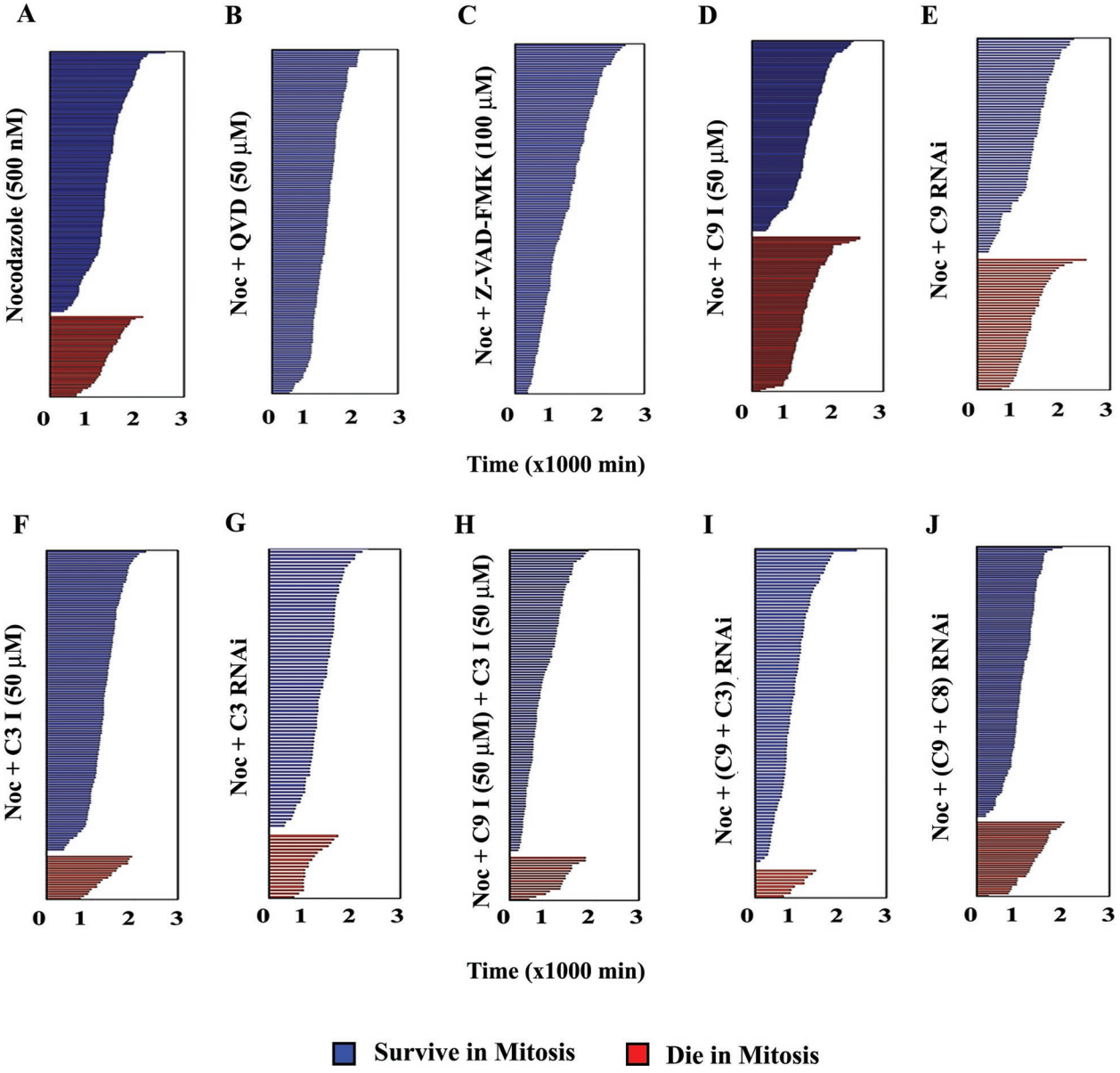


FIGURE 3: Full data sets depicting the duration of mitosis and fate profiles for RPE-1 cells treated with 500 nM nocodazole after depleting or inhibiting various caspases or caspase combinations. The duration each cell spent in mitosis (in minutes) before slipping (blue lines) or dying in mitosis (red lines) is plotted on the *x*-axis, and the conditions for each data set are noted on the side of each plot. A tabulated form of this data can be found in Table S1. See the text for details.

both C3 and C9 before nocodazole treatment: 92% and 87%, respectively, survived mitosis, compared with 76% in nocodazole alone (Figure 3, A and H–I, and Table S1, cf. lanes 1 and 8–9). Depleting or inhibiting just the C3 executioner caspase also increased the number of nocodazole-treated RPE-1 cells that survived mitosis from 76% to, respectively, 81% and 87% (Figure 3, A and F–G, and Table S1, cf. lanes 1 and 6–7). Surprisingly, however, the number of nocodazole-treated cells that survived mitosis was significantly reduced when just C9 was inhibited ($p \leq 0.05$) or depleted ($p \leq 0.05$); compared with nocodazole treatment alone, the numbers dropped from 76% to 55% and 62%, respectively, while at the same time the duration of mitosis (Figure 3, A and D–E, and Table S1, cf. lanes 1 and 4–5) was not appreciably prolonged.

We then repeated this study on Eg5-inhibited cells, which form robust monopolar spindles exhibiting normal microtubule dynamics. (These cells are blocked in mitosis because at any one time ~30% of the kinetochores are not attached to microtubules; see Kapoor *et al.*, 2000.) As for nocodazole, simultaneously depleting C3 and C9 significantly ($p \leq 0.05$) increased the percentage of DME-treated RPE-1 cells that survived to slip through mitosis from 83% to 92% (Figure 4, A and I, and Table S1, cf. lanes 11 and 19), although simultaneously inhibiting both had little effect (Figure 4H and Table S1, lane 18). However, in contrast to nocodazole, we saw no decrease in the viability of Eg5-inhibited RPE-1 cells when C9 was inhibited or depleted; survival under these conditions (82% and 84%, respectively) was similar to DME treatment alone (83%; Figure 4, A and D–E, and Table S1, cf. lanes 11 and 14–15). This suggests that inhibiting or depleting C9 when the MC cannot be satisfied enhances cell lethality in the absence, but not presence, of spindle microtubules. To test this, we determined how selectively inhibiting C9 affects the survival of RPE-1

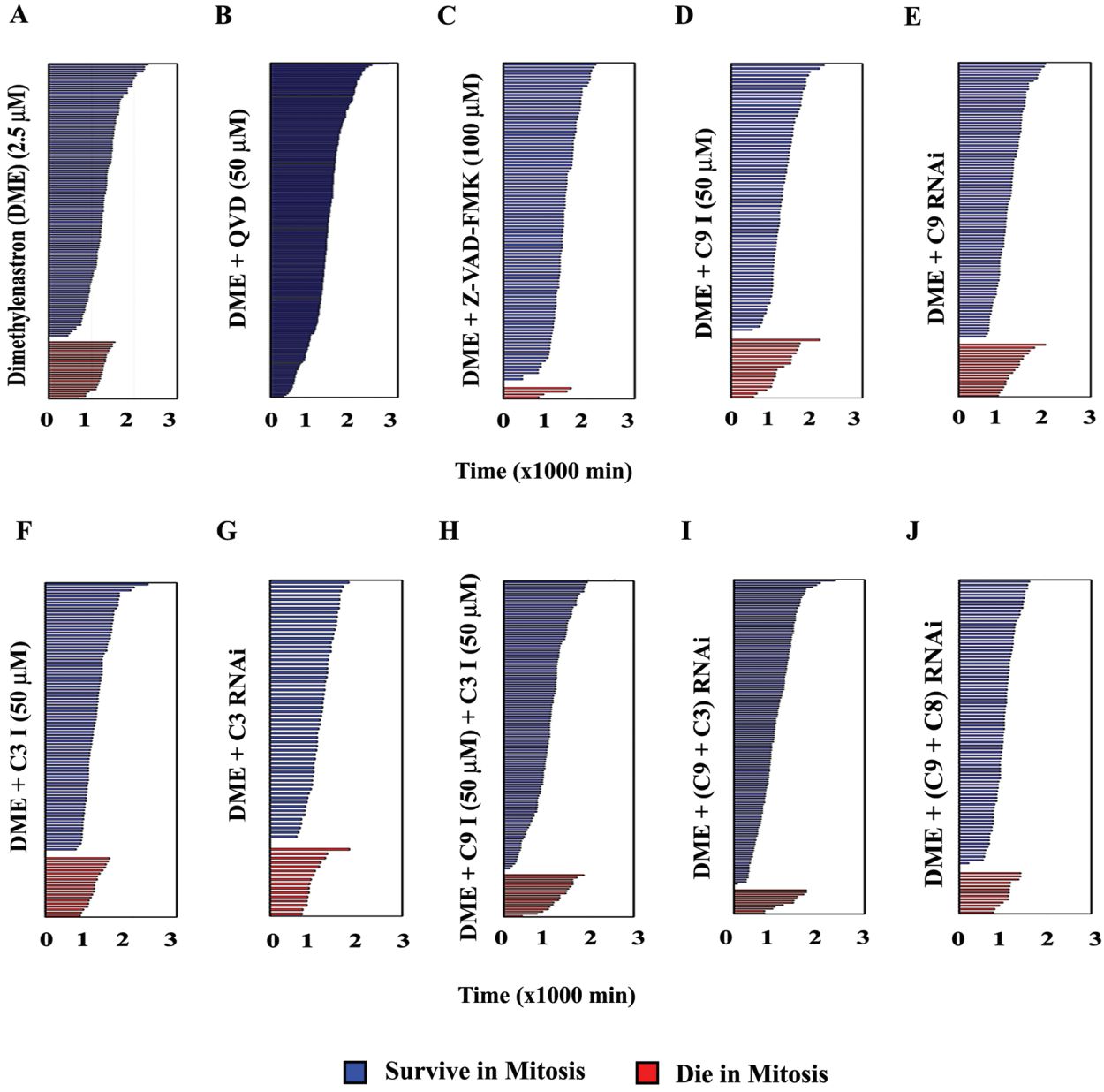


FIGURE 4: Full data sets depicting the duration of mitosis and fate profiles for RPE-1 cells treated with an Eg5 inhibitor (2.5 µM DME) after depleting or inhibiting various caspases or caspase combinations. The duration each cell spent in mitosis (in minutes) before slipping (blue lines) or dying in mitosis (red lines) is plotted on the *x*-axis, and the conditions for each data set are noted on the side of each plot. A tabulated form of this data can be found in Table S1. See the text for details.

cells that enter mitosis in the presence of 500 nM Taxol, which stabilizes spindle microtubules. Under this condition, we found no difference in the number of cells that survived to slip in Taxol alone (117/143 or 82%; see also Shi *et al.*, 2008; Brito and Rieder, 2009) or those treated with both Taxol and our C9 inhibitor (45/55 or 80%).

### Selectively inhibiting certain caspases when the MC cannot be satisfied shortens mitosis

When RPE-1 cells cannot satisfy the MC, they average 21–22 h in mitosis before slipping into the next G1 or dying, and under these conditions treating cells with pan-caspase inhibitors has little effect on the average duration of mitosis prior to slippage. We found that the same was true when we individually inhibited or depleted C3 or C9 (Figures 3, D–G, and 4, D–G, and Table S1, cf. lanes 4–7 and 14–17). Surprisingly, however, the average duration of mitosis in the 87–92% of the cells that slipped was significantly reduced ($p \leq 0.0001$) when both C9 and C3 were simultaneously inhibited or depleted before treating with nocodazole; the duration dropped from 20–22 h to 13–15 h (Figures 3, H–I, and 5A, and Table S1, cf. lanes 8–9). A similar, less dramatic but still significant ($p \leq 0.0001$) result was seen in Eg5-inhibited cells (Figure 5B); when both C9 and C3 were inhibited or depleted, mitosis averaged, respectively, 18 and 16 h, instead of 22 h, and 87–92% survived to slip (Figure 4, A and H–I, and Table S1, cf. lanes 11 and 18–19). Thus, although individually inhibiting or depleting C9 or C3 does not influence the rate at which RPE-1 cells slip through mitosis when the MC cannot be

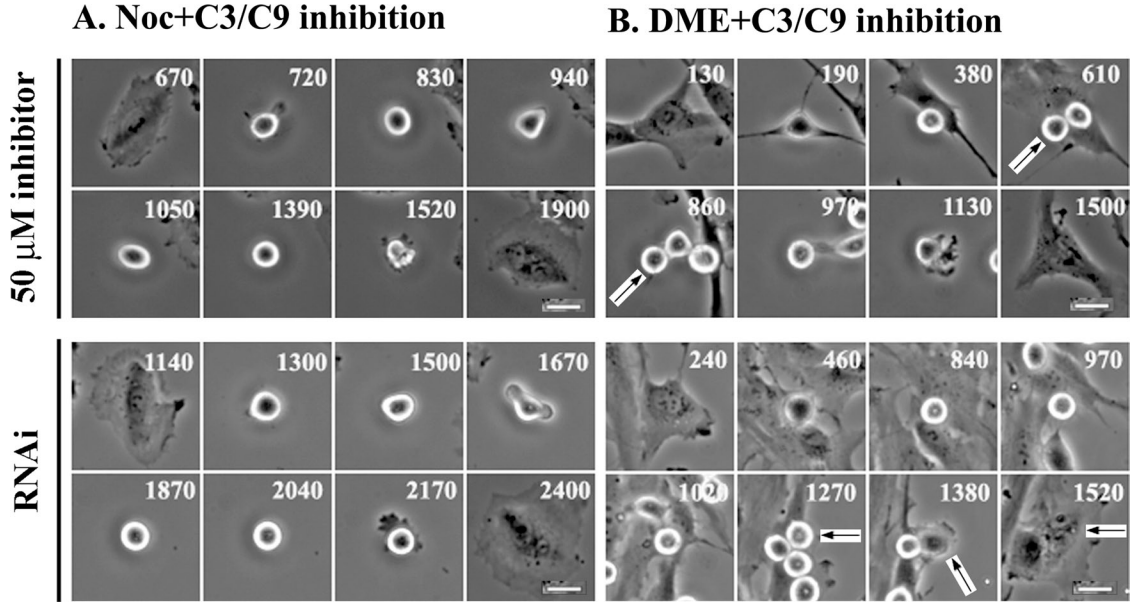


FIGURE 5: Simultaneously inhibiting or depleting C3 and C9 with drugs or RNAi shortens the duration of mitosis in RPE-1 cells treated with nocodazole (A) or Eg5 inhibitors (B). Images from time-lapse recordings of cells entering and exiting mitosis after inhibiting or depleting C3 and C9 before treatment with 500 nM nocodazole (A) or 2.5 μM DME (B). In each sequence, the second frame contained the first evidence of cell rounding and was defined as entry into mitosis. The seventh frame in the sequence contained the first evidence of telophase/membrane blebbing associated with reflattening and was defined as exit from mitosis. In the absence of C3 and C9 activity, RPE-1 cells required, on average, 13–15 h (A) and 16–18 h (B) to slip through mitosis in the absence of microtubules (A) or the presence of a monopolar spindle (B). Time from the start of recording is shown in minutes. Arrows indicate the cell that is followed in subsequent frames. Scale bars: 10 μm.

satisfied, inhibiting C9 and C3 together significantly increases the rate of slippage. It is of interest that under this condition survivability is enhanced (cf. Figure 3A with 3, H–I, Figure 4A with 4, H–I, and Table S1, lane 1 with lanes 8–9 and lane 11 with lanes 18–19), and in the presence of monopolar spindles those cells that died in mitosis did so with normal kinetics (~22 h; Figure 4, A and H–I, and Table S1). The same was true for cells that entered mitosis in nocodazole after C3 and C9 inhibition (Figure 3H, and Table S1, lane 8). Thus inhibiting C9 and C3 promotes survival by accelerating slippage in a subset of RPE-1 cells, while in a smaller subset it has no effect. Finally, accelerated satisfaction of the MC was not unique to inhibiting or depleting C9 and C3, since it also occurred to a lesser degree by simultaneously depleting C9 and C8 (cf. Figures 3, A and J, with 4, A and J, and Table S1, lanes 1 and 10 with 11 and 20).

### Caspase-mediated destruction of BubR1 is not required for mitotic slippage

As noted in the *Introduction*, mitotic slippage in HeLa is reported to occur through the progressive destruction of the MC protein BubR1 (Shin *et al.*, 2003) in a caspase-dependent manner (Baek *et al.*, 2005; Kim *et al.*, 2005, 2008). However, this conclusion is not consistent with our finding that inhibiting caspases does not prolong the duration of mitosis in RPE-1 (Table S1) and other cells when the MC cannot be satisfied (Huang *et al.*, 2010). It also conflicts with an earlier report that BubR1 and other MC proteins are still present on kinetochores of RPE-1 cells that had slipped through mitosis in nocodazole (Brito and Rieder, 2006). To reexamine this issue, we fixed RPE-1 cultures after 22 h in nocodazole, with and without a simultaneous incubation in QVD, and stained them for the presence of BubR1. Since nocodazole-treated RPE-1 average 21 h in mitosis with a SD of ~7 h, we reasoned that cells in the cultures that had slipped within the first 22 h would have done so ≤8 h before fixation. Under both conditions, ~60% of the cells that had slipped through mitosis within 22 h ( identified by the presence of multiple micronuclei) contained numerous BubR1 spots in their micronuclei, while 40% lacked all BubR1 staining (Figure 6). These data reveal that slippage in nontransformed human RPE-1 cells is not preceded by the caspase-mediated destruction of BubR1; although BubR1 does appear to be destroyed several hours after slippage has occurred.

## DISCUSSION

We found that inhibiting caspase activity in toto with pan-caspase inhibitors, or selectively inhibiting/depleting various caspases individually or in combination, has no effect on the timing or fidelity of mitosis in nontransformed human RPE-1 cells. This differs considerably from reports that inhibiting C3 (Swe and Sit, 2000) or -7 (Hashimoto *et al.*, 2008) arrests human cancer (Chang liver and HeLa) cells in mitosis. We believe that the conclusions of these studies are based on methodological artifacts resulting from a prolonged treatment with cytostatic concentrations of pan-caspase inhibitor or to overillumination during confocal imaging. Indeed, cell lines are available that lack C3 (e.g., MCF-7; Janicke, 2009) or C3 and -7 (Lakhani *et al.*, 2006), and viable mice can be produced lacking either caspase (Kuida *et al.*, 1996; Zandy *et al.*, 2005). We also found that treating RPE-1 cultures with pan-caspase inhibitors in the presence of spindle poisons prevented most if not all death in mitosis without prolonging the duration required for mitotic slippage. Similarly, when the MC cannot be satisfied, knocking down or inhibiting C3 or -9 did not delay slippage in RPE-1, but it did lead to an increase in the number of cells that successfully slipped. These data are not consistent with idea that C3 is required for a functional MC (Hsu *et al.*, 2006). Again, either this finding is unique to the Hep3B and HepG2 cells used or, more likely, it is an artifact of the

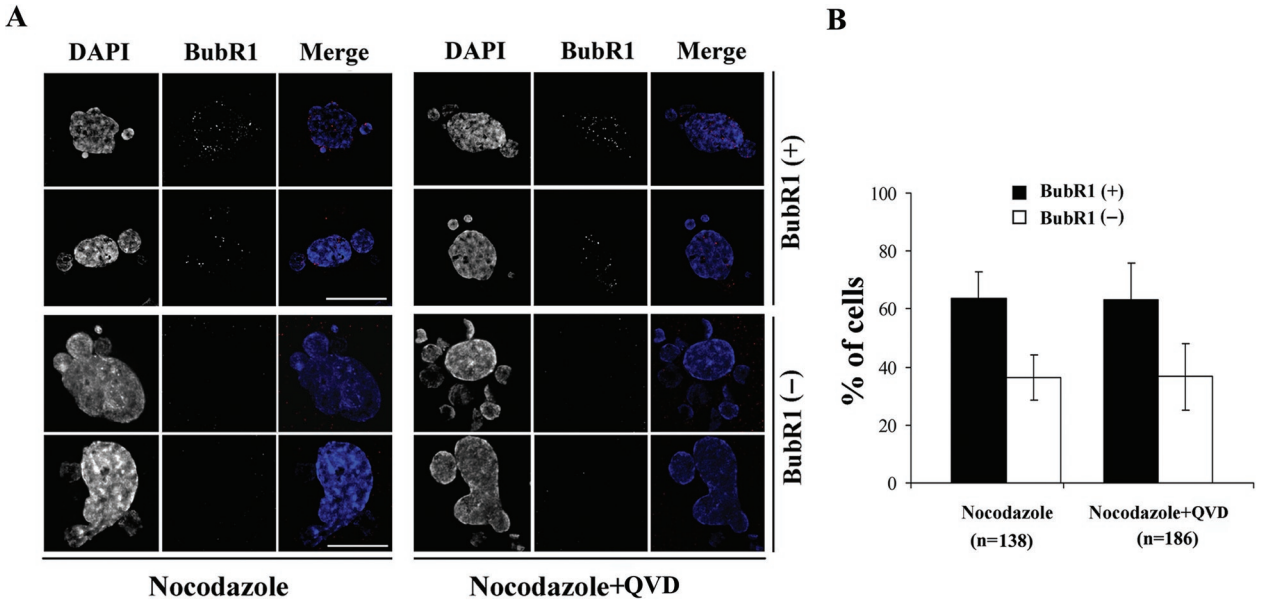


FIGURE 6: BubR1 proteolysis is not a requirement for slippage in RPE-1 cells. (A) BubR1 remains associated with the centromeres of many RPE-1 cells shortly after they have slipped through mitosis in 500 nM nocodazole alone or nocodazole plus 50 μM QVD. Cultures were treated with nocodazole for 22 h before fixation and immunostaining for BubR1. (B) The percentage of BubR1 positive or negative 4N G1 cells in cultures fixed 22 h after treatment with 500 nM nocodazole or nocodazole plus 50 μM QVD. There is no significant difference between the numbers of BubR1-positive nuclei in nocodazole or nocodazole plus QVD-treated cells. n = number of cells. Scale bars: 10 μm.

methodology used. In this regard human, MCF-7 cells have a robust MC even though they lack C3 (Mohan and Panda, 2008; Banerjee *et al.*, 2010).

Biochemical and FACS analyses of exponentially growing HeLa cultures treated with nocodazole led Kim *et al.* (2005) to conclude that pan-caspase inhibitors greatly prolong the duration of mitosis when the MC cannot be satisfied. Similarly, inhibiting caspase activity in nocodazole-treated HeLa significantly prolonged the duration of mitosis in cells that died in mitosis (Brito and Rieder, 2009). Most recently, Gascoigne and Taylor (2008) reported, from live-cell analyses, that "caspase inhibition often prolonged the duration of the mitotic arrest" in several tumor cell lines including RKO, HT29, Calu6, and HCT116. This was also true for one of their nontransformed lines: the average duration of mitosis in nocodazole-treated HME that died in mitosis in the presence of a pan-caspase inhibitor (~10%) was ~60% longer than those (~40%) that died during mitosis in nocodazole only. These data are consistent with the idea that caspase activity is required for timely mitotic slippage. We note, however, that in all of these reports the prolongation of mitosis by pan-caspase inhibitors in cells treated with spindle poisons was seen primarily in those cells that ultimately died in mitosis.

Although there is considerable variation between lines, a large percentage of cells in many tumor lines die in mitosis when the MC cannot be satisfied, because they have lost the ability to slip (i.e., degrade their cyclin B below a threshold level) in a timely manner. A notable example is HeLa: when unsynchronized cultures are treated with an Eg5 inhibitor, the mitotic index (based on phospho-H3 staining) peaks at ~24 h, at which time the negative regulator of apoptosis (X-linked inhibitor of apoptosis protein [XIAP]) begins to disappear, while poly(ADP-ribose)polymerase 1 (PARP1) cleavage (apoptosis) begins, peaking at 38 h (Shi *et al.*, 2008). On the basis of live-cell studies, Gascoigne and Taylor (2008) concluded that 80% of HeLa cells die in mitosis when treated with Eg5 inhibitors. (Their results, which indicated that ~90% of nocodazole-treated HeLa survived mitosis, were due to a nocodazole concentration (100-nM) that was too low to prevent satisfaction of the MC [see Brito *et al.*, 2008].) In previous studies in which 1.5 μM nocodazole was used, we also found that, on average, > 50% of HeLa cells died in mitosis after ~20 h, and under this condition, pan-caspase inhibitors prolonged mitosis in those cells that died in mitosis by ~35% (from an average of 20–27 h; Brito and Rieder, 2009). Importantly, however, the duration of mitosis in those nocodazole-treated HeLa cells that slipped in pan-caspase inhibitors (62%) was similar to those that slipped in nocodazole alone (~20 h). The same result was recently reported by Huang *et al.* (2010), who found no difference in the duration of mitosis in Eg5-inhibited HeLa cells that successfully slipped in the absence or presence of a pan-caspase inhibitor. The most straightforward interpretation of these live-cell studies is that pan-caspase inhibitors prolong mitosis when the MC cannot be satisfied by delaying apoptosis in those cells within the population that have a reduced ability to slip (until, at some point, the cells finally slip or die in mitosis). The percentage of "slippage-challenged" cells varies from a sizable portion in HeLa (and HME; Gascoigne and Taylor, 2008) to very few in RPE-1. It is worth noting that our finding that most RPE-1 slip from mitosis in spindle poisons within 21–22 h, and that pan-caspase inhibitors allow basically all of those that would die in mitosis within this period to slip, is consistent with and expected of a nontransformed genetically "homogenous" and stable cell line in which there is minimal cell-to-cell variability.

The notion that slippage is due to the caspase-mediated destruction of kinetochore-associated BubR1 is based on biochemical analyses of synchronized HeLa populations arrested in mitosis for 24–48 h (Baek *et al.*, 2005; Kim *et al.*, 2005, 2008), and is no longer tenable. When HeLa (and many other types of transformed cells) cannot satisfy the MC they begin to die in mitosis within ~20 h (see preceding paragraph), and synchronized populations of cells that

enter mitosis at the same time can be expected to contain many dying cells when harvested at 24 h, and considerably more at 36 h. Since human Bub1 is cleaved during apoptosis (Perera and Freire, 2005) it is highly likely that the caspase-mediated destruction of BubR1 reported for HeLa cells blocked in mitosis for prolonged periods is not the cause of slippage, but is rather due to apoptosis during mitosis. Indeed, in our live-cell studies, we and others (see Huang *et al.*, 2010) found that when the MC cannot be satisfied pan-caspase inhibitors do not delay slippage, as would be predicted if slippage required caspase activity. Furthermore, although the APC/C-mediated destruction of BubR1 normally occurs as untreated cells exit mitosis, an otherwise functional but nondestructible Bub1 protein (Bub1 and BubR1 are paralogues; see Bolanos-Garcia and Blundell, 2010) has no mitotic phenotype, revealing that BubR1 degradation is not required for mitotic exit (Qi and Yu, 2007). Finally, as seen in Figure 6, when the MC cannot be satisfied, BubR1 is retained on kinetochores as cells slip from mitosis.

When the MC cannot be satisfied, 96–100% of RPE-1 cells survive to slip when caspase activity is inhibited in toto by pan-caspase inhibitors (Figures 3 and 4). We were therefore surprised to find that selectively knocking down or inhibiting C9 before treatment with nocodazole, but not with our Eg5 inhibitor or 500 nM Taxol, significantly ($p \leq 0.05$) increased the number of RPE-1 cells that died in mitosis from 24% to, respectively, 38% and 45%. This suggests that the absence of C9 activity during mitosis is significantly more lethal in cells lacking microtubules than in those that can form monopolar spindles. This difference is not due to the fact that survivin, which is a potent C9 inhibitor (reviewed in Mita *et al.*, 2008), inhibits C9 only when bound to spindle microtubules (Li *et al.*, 1998); when RPE-1 cells enter mitosis in the absence of microtubules (in nocodazole) only 24% die in mitosis, compared with 38–45% that die in nocodazole when C9 is inhibited or depleted (Figure 3, A and D–E). Since both of our pan-caspase inhibitors completely prevented death during mitosis in the absence of microtubules, death during mitosis in nocodazole-treated cells lacking C9 activity appears to be mediated by other caspases. The most likely explanation for our observation is therefore that inhibiting or depleting C9 (but not C3) leads to a more lethal compensatory activation of other initiator caspases, as occurs in mice lacking C9 when challenged with the Fas agonistic antibody Jo2 (Zheng *et al.*, 2000). This explanation is supported by our finding that depleting C9 alone, compared with depleting both C9 and the other initiator caspase (C8) known to be regulated during mitosis (Matthess *et al.*, 2010), reduced nocodazole-induced death in mitosis from ~38% to less than that seen for cells treated with nocodazole alone (22%; Figure 3, E and J, and Table S1, cf. lanes 5 and 10).

Finally, as predicted from the recent literature, we found that simultaneously inhibiting or depleting both C9 and C3 increased the percentage of nocodazole-treated RPE-1 cells that survived mitosis. However, we also found that this treatment significantly reduced the average duration of mitosis in the 92% of the cells that slipped from an average of ~21 h to 13–15 h, and that this was also true for cells treated with our Eg5 inhibitor, although to a lesser extent (from 22 h to 16–18 h). Since slippage results from a slow background APC-mediated ubiquitination and proteolysis of cyclin B (Brito and Rieder, 2006; J. Lee *et al.*, 2010), a lack of C3 and C9 activity somehow increases this rate. Again, this is a surprising result, given that neither of our pan-caspase inhibitors, which prevented most if not all apoptosis during mitosis in nocodazole-treated or Eg5-inhibited cells, altered the rate of slippage. This suggests that inhibiting certain caspase combinations when the MC cannot be satisfied accelerates slippage by modifying the activity of other caspases that, directly or indirectly, promote cyclin B ubiquitination. This could occur, for example, due to modification of the activity of the ACP/C auxiliary factor, UBE2S, which promotes slippage in spindle poisons by enhancing ubiquitin chain elongation on cyclin B (Garnett *et al.*, 2009). However, regardless of the explanation, it is clear from our data that inhibiting or depleting caspases individually or in combination when the MC cannot be satisfied can lead to a different outcome than when the activity of all caspases is inhibited.

## MATERIALS AND METHODS

### Cell culture and reagents

Stock cultures of hTERT-RPE-1 (RPE-1) cells were maintained at 37°C in a humid 5% $CO_2$ environment in DMEM supplemented with 10% fetal bovine serum (FBS). Z-VAD (OMe)-FMK (#627610), its nonfunctional analogue Z-Phe-Ala-FMK (#342000), QVD (#551476), C3 inhibitor (#235420), C8 inhibitor (#218840), C9 inhibitor (#218761), and DME (#324622) were all purchased from Calbiochem (San Diego, CA). Nocodazole and Taxol were purchased from Sigma-Aldrich (St. Louis, MO). Reagents were diluted in dimethyl sulfoxide (DMSO) and used at the concentrations indicated in the text and Tables 1 and S1. Before experimentation, RPE-1 cells were seeded from stock flasks onto 24-$mm^2$ coverslips and incubated at 37°C for at least 16 h.

### Live-cell microscopy

Coverslip cultures were assembled into Rose chambers containing phenol-free L-15 medium with 10% FBS, and incubated at 37°C for 2–4 h (Khodjakov and Rieder, 2006). Before imaging, drugs were added to the medium at the concentrations noted in the Tables 1 and S1. Caspase inhibitors were added to the medium ~1 h before adding nocodazole or DME (Brito *et al.*, 2008).

For studies on the duration of mitosis, time-lapse images were captured at 1-min intervals for 24 h or at 10-min intervals for 48 h at 37°C with either a 10× 0.3 numerical aperture (NA) or 20× 0.5 NA Plan Fluor objective lens mounted on a Nikon Eclipse TE2000-U microscope equipped with shuttered Micromax (Roper Scientific, Tucson, AZ) or Orca ER (Hamamatsu Photonics, Bridgewater, NJ) cameras. Time-lapse image sequences were compiled with Image J (http://rsb.info.nih.gov/ij/download.html). All timing data were collected on two to three coverslips filmed on different days.

### RNAi and immunoblotting

Synthetic double-stranded siRNAs for C3 and/or -9 and C8 and -9 were used. For this experiment, we used the ON-TARGET plus SMART pool sequences provided by Dharmacon (Thermo Scientific, Lafayette, CO): C3 (#L-004307–00), C8 (#L-003466–00), and C9 (#L-003309–00), respectively. We also used ON-TARGET plus Non-Targeting Pool (Dharmacon, #D-001810–10) as a scrambled control. Targeted duplexes were transfected at 100 nM with oligofectamine (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. In some instances, RPE-1 cells were harvested after a 48-h treatment with siRNAs, or the nonsense control, and lysed with 1× sample buffer (62.5 mM Tris, pH 6.8, 2% SDS, 50 mM dithiothreitol [DTT], 10% glycerol, 0.01% bromophenol blue) for subsequent immunoblotting. The primary antibodies used were mouse anti–α-tubulin (T-5168, Sigma-Aldrich), rabbit anti–C3 (#9662), C8 (#4790), and C9 (#9502), all from Cell Signaling (Beverly, MA).

### Indirect immunofluorescence

Coverslip cultures were briefly washed with phosphate-buffered saline (PBS) prior to fixation in cold 100% methanol (−20°C) and

permeabilized with 0.5% Triton X-100 in PBS as previously detailed (K. Lee *et al.*, 2010). Our primary rabbit anti-BubR1 antibody was a kind gift from Tim Yen (Fox Chase, Philadelphia, PA); secondary rabbit antibody was Alexa Fluor 568 (Invitrogen). Image stacks were acquired and deconvolved on a Delta Vision System (Applied Precision, Issaquah, WA) centered on an Olympus IX70 microscope equipped with a CM350 Photometrics camera.

To determine the relative frequencies of mitotic stages, we treated growing RPE-1 coverslip cultures with either a pan-caspase inhibitor (QVD) or a C9 inhibitor. After 16 h in either drug, the cultures were fixed and stained with DAPI (Invitrogen). The population of mitotic cells within each was then scored against untreated cultures for the number of cells in prometaphase, metaphase, or anaphase. This experiment was conducted three times on three different days.

For mitotic index studies, we treated growing RPE-1 coverslip cultures with 50 μM of the C9 inhibitor. After 6 h, one untreated and one drug-treated coverslip were fixed and stained with DAPI. The number of prometaphase, metaphase, and anaphase/telophase cells within a random population of 500 cells per coverslip was then determined. The experiment was repeated the next day, and the results of the two separate experiments were pooled to obtain the total number of mitotic figures per 1000 cells for each condition.

For our studies on lagging chromosomes, we compared untreated coverslip cultures with those treated with QVD. After 16 h of QVD treatment, we fixed a single control and a single experimental coverslip, and stained each with DAPI. These were then examined to determine the number of anaphase cells, and the percentage that contained lagging chromosomes, on each coverslip. We then repeated this experiment 2 more times over a 2-d period and pooled the data.

To determine the percentage of BubR1-positive or -negative cells after slippage, we first treated RPE-1 coverslip cultures with QVD, and added nocodazole1 h later. Controls were exposed only to nocodazole. We fixed the coverslip cultures after 22 h and stained them with DAPI and for the indirect immunofluorescence presence of BubR1. We counted those cells that had slipped through mitosis and that contained or lacked BubR1-staining foci in their multiple micronuclei. This experiment was conducted two times on two different days.

## ACKNOWLEDGMENTS

C.L.R. thanks the National Institute of General Medical Sciences (Grant 40198), National Institutes of Health, and the U.S. taxpayers for generously supporting his lab-based research efforts during the past 30 years.

## REFERENCES

Allan LA, Clarke PR (2007). Phosphorylation of caspase-9 by CDK1/cyclin B protects mitotic cells against apoptosis. Mol Cell 26, 301–310.

Andersen JL, Johnson CE, Freel CD, Parrish AB, Day JL, Buchakjian MR, Nutt LK, Thompson JW, Moseley MA, Kornbluth S (2009). Restraint of apoptosis during mitosis through interdomain phosphorylation of caspase-2. EMBO J 28, 3216–3227.

Baek K-H, Shin H-J, Jeong S-J, Park J-W, McKeon F, Lee C-W, Kim C-M (2005). Caspases-dependent cleavage of mitotic checkpoint proteins in response to microtubule inhibitor. Oncol Res 15, 161–168.

Banerjee M, Singh P, Panda D (2010). Curcumin suppresses the dynamic instability of microtubules, activates the mitotic checkpoint and induces apoptosis in MCF-7 cells. FEBS J 277, 3437–3448.

Brenner D, Mak TW (2009). Mitochondrial cell death effectors. Curr Opin Cell Biol 21, 871–877.

Bolanos-Garcia M, Blundell TL (2011). Bub1 and BubR1: multifaceted kinases of the cell cycle. Trends Biochem Sci 36, 141–150.

Brito DA, Rieder CL (2006). Mitotic checkpoint slippage in humans occurs via cyclin B destruction in the presence of an active checkpoint. Curr Biol 16, 1194–1200.

Brito DA, Rieder CL (2009). The ability to survive mitosis in the presence of microtubule poisons differs significantly between human nontransformed (RPE-1) and cancer (U2OS, HeLa) cells. Cell Motil Cytoskel 66, 437–447.

Brito DA, Yang Z, Rieder CL (2008). Microtubules do not promote mitotic slippage when the spindle assembly checkpoint cannot be satisfied. J Cell Biol 182, 623–629.

Caserta TM, Smith AN, Gultice AD, Reedy MA, Brown TL (2003). Q-VD-OPh, a broad spectrum caspase inhibitor with potent antiapoptotic properties. Apoptosis 8, 345–352.

Chauvier D, Ankri S, Charriaut-Marlangue C, Casimir R, Jacotot E (2007). Broad-spectrum caspase inhibitors: from myth to reality? Cell Death Differ 14, 387–391.

Eckelman BP, Salvesen GS, Scott FL (2006). Human inhibitor of apoptosis proteins: why XIAP is the black sheep of the family. EMBO Rep 7, 988–994.

Fuentes-Prior P, Salvesen GS (2004). The protein structures that shape caspase activity, specificity, activation and inhibition. Biochem J 384, 201–232.

Garnett MJ, Mansfeld J, Godwin C, Matsusaka T, Wu J, Russell P, Pines J, Venkitaraman AR (2009). UBE2S elongates ubiquitin chains on APC/C substrates to promote mitotic exit. Nat Cell Biol 11, 1363–1369.

Gartner M, Sunder-Plassman N, Seiler J, Utz M, Vernos I, Surrey T, Giannis A (2005). Development and biological evaluation of potent and specific inhibitors of mitotic kinesin Eg5. ChemBioChem 6, 1173–1177.

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

Harley ME, Allan LA, Sanderson HS, Clarke PR (2010). Phosphorylation of Mcl-1 by CDK1-cyclin B1 initiates its Cdc20-dependent destruction during mitotic arrest. EMBO J 29, 2407–2420.

Hashimoto T, Yamauchi L, Hunter T, Kikkawa U, Kamada S (2008). Possible involvement of caspase-7 in cell cycle progression at mitosis. Genes Cells 13, 609–621.

Hsu S-L, Yu C-TR, Yin S-C, Tang M-J, Tien A-C, Wu Y-M, Huang C-YF (2006). Caspase 3, periodically expressed and activated at G2/M transition, is required for nocodazole-induced mitotic checkpoint. Apoptosis 11, 765–771.

Huang H-C, Mitchison TJ, Shi J (2010). Stochastic competition between mechanistically independent slippage and death pathways determines cell fate during mitotic arrest. PLoS One 5, 12e15724.

Janicke RU (2009). MCG-7 breast carcinoma cells do not express caspase-3. Breast Cancer Res Treat 117, 219–221.

Kapoor TM, Mayer TU, Coughlin ML, Mitchson TJ (2000). Probing spindle assembly mechanisms with monastrol, a small molecule inhibitor of the mitotic kinesin, Eg5. J Cell Biol 150, 975–988.

Khodjakov A, Rieder CL (2006). Imaging the division process in living tissue culture cells. Methods 38, 2–16.

Kim H, Farris J, Christman SA, Kong B-W, Foster LK, O'Grady SM, Foster DN (2002). Events in the immortalizing process of primary human mammary epithelial cells by the catalytic subunit of human telomerase. Biochem J 365, 765–772.

Kim M, Liao J, Dowling ML, Voong KR, Parker SE, Wang S, El-Deiry WS, Kao GD (2008)). TRAIL inactivates the mitotic checkpoint and potentiates death induced by microtubule-targeting agents in human cancer cells. Cancer Res 68, 3440–3449.

Kim M, Murphy K, Liu F, Parker SE, Dowling ML, Baff W, Kao GD (2005). Caspase-mediated specific cleavage of BubR1 is a determinant of mitotic progression. Mol Cell Biol 25, 9232–9248.

Kozielski F, Skoufias DA, Indorato RL, Saoudi Y, Jungblut PR, Hustoft HK, Strozynski M, Thiede B (2008). Proteome analysis of apoptosis signaling by S-trityl-L-cysteine, a potent reversible inhibitor of human mitotic kinesin Eg5. Proteomics 8, 289–300.

Kuida K, Zheng TS, Na S, Kuan C-Y, Yang D, Karasuyama H, Rakic P, Flavell RA (1996). Decreased apoptosis in the brain and premature lethality in CPP32-deficient mice. Nature 384, 368–372.

Lakhani SA, Masud A, Kuida K, Porter GA, Booth CJ, Mehal WZ, Inayat I, Flavell RA (2006). Caspases 3 and 7: key mediators of mitochondrial events of apoptosis. Science 311, 847–851.

Lanni JS, Jacks T (1998). Characterization of the p53-dependent postmitotic checkpoint following spindle disruption. Mol Cell Biol 18, 1055–1064.

Lee J, Kim JA, Margolis RL, Fotedar R (2010). Substrate degradation by the anaphase promoting complex occurs during mitotic slippage. Cell Cycle 9, 1792–1801.
Lee K, Kenny AE, Rieder CL (2010). P38 mitogen-activated protein kinase activity is required during mitosis for timely satisfaction of the mitotic checkpoint but not for the fidelity of chromosome segregation. Mol Biol Cell 21, 2150–2160.
Li F, Ambrosini G, Chu EY, Plescia J, Tognin S, Marchisio PS, Altieri DC (1998). Control of apoptosis and mitotic spindle checkpoint by survivin. Nature 396, 580–584.
Matthess Y, Raab M, Sanhaji M, Lavrik KN, Strebhardt K (2010). Cdk1/cyclin B1 controls Fas-mediated apoptosis by regulating caspase-8 activity. Mol Cell Biol 30, 5726–5740.
Mita AC, Mita MM, Nawrocki ST, Giles FJ (2008). Survivin: key regulator of mitosis and apoptosis and novel target for cancer therapeutics. Clin Cancer Res 14, 5000–5005.
Mohan R, Panda D (2008). Kinetic stabilization of microtubule dynamics by estramustine is associated with tubulin acetylation, spindle abnormalities, and mitotic arrest. Cancer Res 68, 6181–6189.
Perera D, Freire R (2005). Human spindle checkpoint kinase Bub1 is cleaved during apoptosis. Cell Death Differ 12, 827–830.
Peterson D, Lee J, Lei XC, Forrest WF, Davis DP, Jackson PK, Belmont LD (2010). A chemosensitization screen identifies TP53RK, a kinase that restrains apoptosis after mitotic stress. Cancer Res 70, 6325–6335.
Pop C, Salvesen GS (2009). Human caspases: activation, specificity, and regulation. J Biol Chem 284, 21777–21781.
Qi W, Yu H (2007). KEN-box-dependent degradation of the Bub1 spindle checkpoint kinase by the anaphase promoting complex/cyclosome. J Biol Chem 282, 3672–3679.
Rambhatla L, Chiu CP, Glickman RD, Rowe-Rendleman C (2002). In vitro differentiation capacity of telomerase immortalized human RPE cells. Invest Ophthalmol Vis Sci 43, 1622–1630.
Rensen WM, Roscioli E, Tedeschi T, Mangiacasale R, Ciciarello M, Di Gioia AS, Lavia P (2009). RanBP1 downregulation sensitizes cancer cells to taxol in a caspase-3-dependent manner. Oncogene 28, 1748–1758.
Rieder CL, Maiato H (2004). Stuck in mitosis or passing through: what happens when cells cannot satisfy the spindle assembly checkpoint. Dev Cell 7, 637–651.
Shi J, Orth JD, Mitchison T (2008). Cell type variation in response to antimitotic drugs that target microtubules and kinesin-5. Cancer Res 68, 3269–3276.
Shin HJ *et al.* (2003). Dual roles of human BubR1, a mitotic checkpoint kinase, in the monitoring of chromosomal instability. Cancer Cell 4, 483–497.
Swe M, Sit KH (2000). zVAD-fmk and DEVD-cho induce late mitosis arrest and apoptotic expressions. Apoptosis 5, 29–36.
Tao W, South VJ, Diehl RE, Davide JP, Sepp-Lorenzino L, Fraley ME, Arrington KL, Lobell RB (2007). An inhibitor of the kinesin spindle protein activates the intrinsic apoptotic pathway independently of p53 and de novo protein synthesis. Mol Cell Biol 27, 689–698.
Terrano DT, Upreti M, Chambers TC (2010). Cyclin-dependent kinase 1-mediated Bcl-xL/Bcl-2 phosphorylation acts as a functional link coupling mitotic arrest and apoptosis. Mol Cell Biol 30, 640–656.
Vijapukar U, Wang W, Herbst R (2007). Potentiation of kinesin spindle protein inhibitor-induced cell death by modulation of mitochondrial and death receptor apoptotic pathways. Cancer Res 67, 237–245.
Xu M, Masakatsu T, Oikawa K, Tanaka M, Nishi H, Isaka K, Kudo M, Kuroda M (2009). USP15 plays an essential role for caspase-3 activation during paclitaxel-induced apoptosis. Biochem Biophys Res Commun 388, 366–371.
Zandy AJ, Lakhani S, Zheng T, Flavell RA, Bassnett S (2005). Role of the executioner caspases during lens development. J Biol Chem 280, 30263–30272.
Zheng TS, Hunot S, Kuida K, Momoi T, Srinivasan A, Nicholson EW, Lazebnik Y, Flavell RA (2000). Deficiency in caspase-1 or caspase-3 induces compensatory caspase activation. Nature Med 6, 1241–1247.