

Activation of the apoptotic pathway during prolonged prometaphase blocks daughter cell proliferation

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ABSTRACT When untransformed human cells spend >1.5 h in prometaphase under standard culture conditions, all daughters arrest in G1 despite normal division of their mothers. We investigate what happens during prolonged prometaphase that leads to daughter cell arrest in the absence of DNA damage. We find that progressive loss of anti-apoptotic MCL-1 activity and oxidative stress act in concert to partially activate the apoptosis pathway, resulting in the delayed death of some daughters and senescence for the rest. At physiological oxygen levels, longer prometaphase durations are needed for all daughters to arrest. Partial activation of apoptosis during prolonged prometaphase leads to persistent caspase activity, which activates the kinase cascade mediating the post-mitotic activation of p38. This in turn activates p53, and the consequent expression of p21 stops the cell cycle. This mechanism can prevent cells suffering intractable mitotic defects, which modestly prolong mitosis but allow its completion without DNA damage, from producing future cell generations that are susceptible to the evolution of a transformed phenotype.

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INTRODUCTION

The mitotic checkpoint maintains genomic integrity by blocking the metaphase–anaphase transition until all sister chromatids attach to opposite spindle poles (reviewed in Musacchio and Salmon [2007] and Lara-Gonzalez *et al.* [2012]). However, some problems in spindle assembly or mitotic progression eventually allow checkpoint satisfaction and could result in a completed but defective mitosis. For example, in response to low concentrations of microtubule targeting agents the checkpoint becomes satisfied after many hours even though the spindle is short and/or multipolar (Brito *et al.*, 2008; Yang *et al.*, 2009). For cells held in G2 and then released into mitosis, prolonging prometaphase with the Eg5 inhibitor monastrol can

lead to centrosome fragmentation and mother–daughter centriole separation (Karki *et al.*, 2017). Reduced spindle size can compromise cleavage completion, and spindle multipolarity can lead to unequal chromosome distribution. In real life, environmental toxins, radiation, or chemotherapeutic agents could produce spindle defects that result in completed but inaccurate mitoses. Also, environmental stresses that prolong prometaphase without diminishing spindle assembly/function can lead to cohesion fatigue that compromises genomic stability (reviewed in Gorbsky [2013]). Whether or not the cell has a way to protect against such mitotic defects that evade the mitotic and DNA damage checkpoints has not been clear.

Once the mitotic checkpoint is satisfied and the cell completes mitosis, it would be logical for the daughter cells to proliferate regardless of prometaphase duration. However, when untransformed cells are continuously treated with microtubule targeting agents, they remain in prometaphase for up to 40 h and after slipping into interphase arrest in G1 (Cross *et al.*, 1995; Minn *et al.*, 1996; Lanni and Jacks, 1998; Rieder and Maiato, 2004; Brito and Rieder, 2006; Demidenko *et al.*, 2008). Similarly, transient (~16–20 h) prolongations of prometaphase allow completion of mitosis but the daughter cells nevertheless arrest (Ciciarello *et al.*, 2001; Tritarelli *et al.*, 2004). For such grossly prolonged mitoses, daughter cell arrest can be attributed to p53 activation following DNA damage starting after ~16 h in prometaphase (Orth *et al.* [2012] also see Dalton *et al.* [2007],

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Abbreviations used: FITC, fluorescein isothiocyanate; JNK, c-Jun N-terminal kinase; Mcl-1, myeloid cell leukemia factor 1; RPE, retinal pigment epithelium.

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Quignon *et al.* [2007], and Hayashi *et al.* [2012]). This DNA damage results from the sublethal activation of the apoptosis pathway during prometaphase and consequent caspase-activated DNase activity (Orth *et al.*, 2012). For transformed cells held continuously in mitosis with microtubule agents such as taxol, robust activation of the apoptosis pathway during prometaphase kills them during mitosis if they do not slip into interphase too soon (reviewed in Topham and Taylor [2013]).

For untransformed human cells, we investigated the relationship between prometaphase duration of mother cells and the proliferative capacity of daughter cells under standard culture conditions (Uetake and Sluder, 2010). Daughter cells proliferated if prometaphase lasted <1.5 h. However, when cells spent >1.5 h in prometaphase, the daughters remained viable but arrested in G1 despite the normal divisions of their mothers. This daughter cell arrest occurred after prometaphase durations less than those needed for apoptotic death in mitosis for cells constitutively arrested in mitosis (Gascoigne and Taylor, 2008; Topham and Taylor, 2013; Sloss *et al.*, 2016). The G1 arrest we observed was p38 and p53 dependent and enforced by p21 expression. The basis for the activation of a p38–p53 response after modest prolongation of prometaphase was a mystery, because we ruled out DNA damage, a high incidence of lagging chromosomes, prolonged activity of the mitotic checkpoint, and p53 accumulation during mitosis for prometaphase durations lasting up to 6 h.

When we prolonged prometaphase for >1.5 h and transiently inhibited p38 activity for at least 3 h just after mitosis, the daughter cells progressed through interphase and divided again. Remarkably, all granddaughter cells arrested in G1, even though they were born from mitoses of normal duration (Uetake and Sluder, 2010). Thus, after more than ~1.5 h in prometaphase, there is an irreversible change in the properties of the daughter cells that cannot be “erased” by progression through interphase and mitosis.

The purpose of the present study is to gain insight into the basis for the DNA damage and mitotic defect independent G1 arrest of daughter cells after moderate prolongation of prometaphase. We investigate what happens during modestly prolonged prometaphase that leads to a durable change in the properties of daughter cells and the nature of that change.

RESULTS

We used hTERT RPE1 cells, untransformed human cells that show a qualitatively identical response to prolonged prometaphase as primary human fibroblasts (Uetake and Sluder, 2010). We used 0.08 μ M nocodazole to diminish spindle size and interfere with chromosome attachment, thereby prolonging mitotic checkpoint activity. No cells slipped out of mitosis during any of the nocodazole treatments. The G1 arrest of daughter cells after prolonged prometaphase is not nocodazole specific; monastrol, taxol, and MG132 produced qualitatively similar results (Uetake and Sluder, 2010).

Death and senescence after prolonged prometaphase

Since grossly prolonged prometaphase leads to the partial activation of the apoptosis pathway (Orth *et al.*, 2012), we tested whether this also holds true for moderate prolongations of prometaphase (up to 6 h) that do not lead to DNA damage under standard culture conditions (Uetake and Sluder, 2010). We started by following the long-term fate of individual daughter cells born of mothers spending up to 6 h in prometaphase. Asynchronous cultures were treated with nocodazole for 4 h and mitotic shake-off cells were kept in nocodazole for another 2 h. Thus, all cells spent 2–6 h in prometaphase, times sufficient to give a G1 arrest of all daughters (Uetake and Sluder, 2010). For 136 daughters continuously filmed for 303 h

(almost 13 d), 69/136 (51%) eventually died (dots above the time line, Figure 1A), almost all before the culture became confluent at ~240 h (asterisk above time line) due to the proliferation of 1% of the cells starting at 72 h (Uetake and Sluder, 2010). This resumption of proliferation in a few cells could be due to reversal of the apoptotic program (Tang *et al.*, 2012). Figure 1B shows two examples of pronounced blebbing activity followed by cell death. To control for handling and observation conditions we treated asynchronous cells with nocodazole for 30 min, shook off mitotic cells, and then continuously followed them after drug washout. All 40 daughters proliferated, and after the culture became confluent at ~120 h (asterisk under the time line), only 15/1776 cells (0.8%) present at the end of the film runs had died (Figure 1A, dots under the time line).

For daughters of mothers held 2–6 h in prometaphase, 68/109 cells (62%) showed surface exposure of phosphatidylserine by 18 h after nocodazole removal, indicative of early-stage apoptosis as detected with fluorescein isothiocyanate (FITC)-tagged annexin V (reviewed in Krysko *et al.* [2008]) (Figure 1C, all cells). By 96 h all of the annexin V–positive cells had propidium iodide positive nuclei indicating surface membrane permeability in later stage apoptosis. For daughters still living at 72 h, 228/242 (94%) exhibited β -galactosidase staining, indicative of senescence (Figure 1D, first panel, and middle panel for 96 h), unlike any of 312 cells in the control culture treated for 30 min with nocodazole (Figure 1D, right-hand panel). Together these results reveal that prometaphase durations of 6 h or less lead to the activation of the apoptosis pathway but not in an immediately lethal manner.

MCL-1 activity loss during prolonged prometaphase

We next investigated the basis for the partial activation of the apoptosis pathway during prometaphase. The experimental platform used is described in Uetake and Sluder (2010) and here under *Materials and Methods*. Briefly, we prolonged prometaphase to varying extents in mother cells and followed the proliferative capacity of their daughters. Asynchronous cultures were treated for 6 h with 0.08 μ M nocodazole to diminish spindle assembly and maintain mitotic checkpoint activation. Fields of cells were continuously followed so that the times when individual cells entered mitosis were known. Those entering mitosis early spent more time in prometaphase than those entering mitosis near the end of the 6-h treatment. After drug washout, the same cells were continuously followed to determine the proliferative capacity of their daughters. By following individual cells, we precisely knew their prometaphase durations and the behavior of their daughters. All experiments each involved multiple preparations and time-lapse runs conducted on separate days.

The consequences of prolonging prometaphase without additional intervention are shown in Figure 2A. This we call the “basic” experiment. Each vertical bar represents a daughter cell and the height of the bar indicates the duration of prometaphase for its mother cell. Daughters that proliferated are shown as light-colored bars and those that arrested in G1 are shown as dark bars. Some bars are single because one of the two daughter cells crawled off the field of view. The vertical dashed line indicates the prometaphase duration of the mother cells (1.5 h) beyond which all daughters arrest in G1. Thus, the temporal tolerance of RPE1 cells for prolonged prometaphase is 1.5 h under standard culture conditions. Prometaphase in control cells averages 18 min (range 9–30 min, $n = 117$).

The expression and hence activity levels of the antiapoptotic protein MCL-1 gradually decline during prometaphase in cells constitutively held in mitosis with nocodazole or Taxol (Harley *et al.*, 2010; Wertz *et al.*, 2011; Sloss *et al.*, 2016). We tested whether

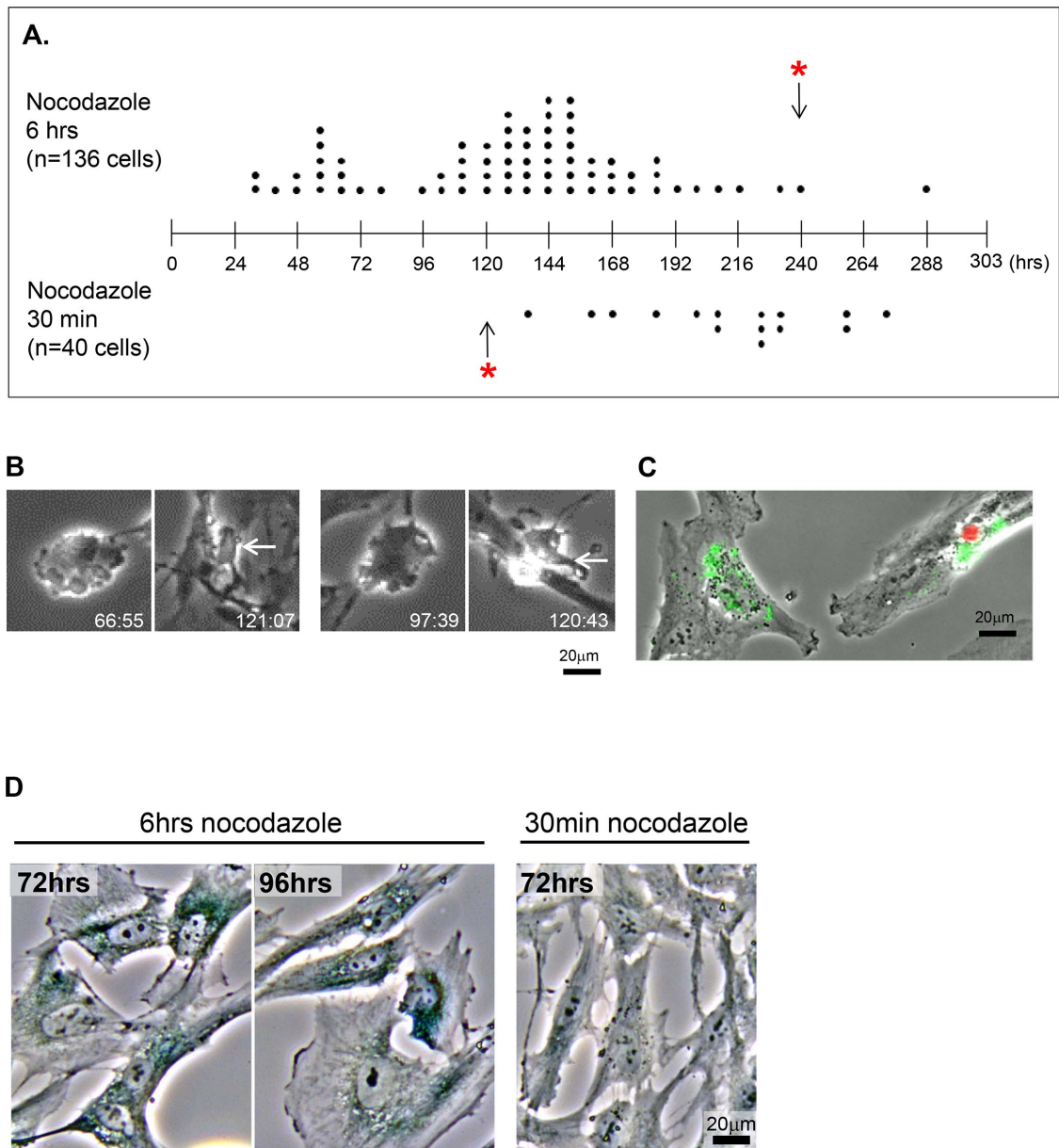


FIGURE 1: (A) Times of individual daughter cell death after prolonged prometaphase. Points above the time line: for daughters born of 136 mothers spending 2–6 h in prometaphase, 69/136 (51%) died, and all but one died before the preparation became confluent (asterisk) due to the proliferation of a few cells starting at 72 h. Points below the time line: for daughters born of 40 mothers treated with nocodazole for 30 min, all daughters proliferated and by the end of the film runs 15/1776 (0.8%) of the progeny died, all after the culture became confluent (asterisk). Time line is hours after removal of nocodazole. (B) Two examples of the death of daughters born of mothers spending 2–6 h in prometaphase. First frames show blebbing activity and the second frames show terminal compaction of the same cells (arrows). Times are hh:mm after nocodazole removal. Phase contrast images, bar = 20 μ m. (C) For daughters born of mothers held 2–6 h in prometaphase, evidence for activation of the apoptotic pathway at 18 h after nocodazole removal as detected with FITC-tagged annexin V (green contrast) for surface phosphatidylserine. Of the annexin V–positive cells, 14/68 (21%) show propidium iodide PI–positive nuclei (red contrast), indicating surface membrane permeability during later stage apoptosis (right-hand cell). Phase contrast/fluorescence image, bar = 20 μ m. (D) Senescence in some G1–arrested daughter cells born of mothers held 2–6 h in prometaphase. First and second frames show examples β –galactosidase positive cells still alive at 72 and 96 h after nocodazole removal. Third frame, none of the daughter cells born of mothers treated with nocodazole for 30 min are β –galactosidase positive at 72 h. Phase contrast images, bar = 20 μ m.

reduction of MCL-1 activity influences how long mother cells must spend in prometaphase before the future daughter cells become committed to arrest in G1. We partially diminished MCL-1 activity with the specific small molecule inhibitor MIM1 at a concentration

that did not kill control cells or change their cell-cycle timing. Our rationale was that partial diminishment of MCL-1 activity should reveal its influence on determining the range of prometaphase durations beyond which daughter cells arrested in G1. At 10 μ M

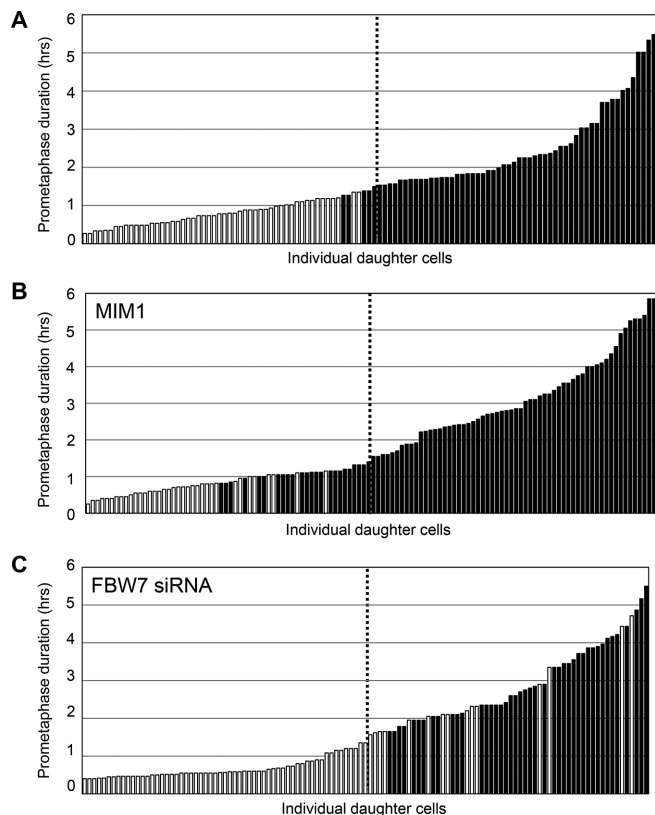


FIGURE 2: (A) Relationship between prometaphase duration and daughter cell proliferation under standard culture conditions—the “basic” experiment (redrawn from Figure 1B of Uetake and Sluder [2010]). Asynchronous cultures were treated with nocodazole for 6 h and entry of individual cells into mitosis followed. After drug washout, daughters of previously followed mothers were continuously followed. Each vertical bar represents a daughter cell remaining in the field of view and the height of the bar indicates the prometaphase duration for its mother cell. The bars are ordered by the duration of prometaphase for the mother cells. Daughters that proliferated are shown as light-colored bars, and those that arrested in G1 are shown as dark colored bars. The vertical dashed line indicates the prometaphase duration of the mother cells (90 min) beyond which all daughter cells arrested in G1. (B) Partial inhibition of MCL1 activity during prometaphase reduces the temporal tolerance for prolonged prometaphase. Asynchronous cultures were treated with nocodazole plus MIM1 for 6 h and both drugs washed out. Significantly fewer daughters born of mothers spending <1.5 h in prometaphase proliferated relative to the basic experiment (A): $p = 0.0019$. For the daughters born of mothers spending >1.5 h in prometaphase, there was no significant increase in the proportion of cells that proliferated ($p = 1.0$). (C) Knockdown of the F-box protein FBW7 allows some daughter cells to proliferate even though their mothers spent up to 4.6 h in prometaphase. Forty-eight hours after siRNA transfection, asynchronous cultures were treated with nocodazole for 6 h and the progeny of individual mother cell were continuously followed. For three pairs of daughter cells born of mothers spending >1.5 h in prometaphase, one daughter proliferated while its sister arrested. Significantly more daughters born of mothers spending >1.5 h in prometaphase proliferated relative to the basic experiment (A): $p = 0.00012$. For the daughters born of mothers spending <1.5 h in prometaphase, there was no significant decrease in the proportion of cells that proliferated ($p = 1.0$).

continuous exposure (IC50 4.8 μM ; Cohen *et al.* [2012]), cells repeatedly cycled with normal morphology and timing (average 19 h, range 18–23 h, $n = 41$ vs. average of 20 h, range 16–26 h, $n = 40$ for

untreated cells). This equivalency indicates that the drug per se, at the concentration used, does not lead to a G1 arrest after normal mitosis or have obvious pleiotropic activity.

We applied 10 μM MIM1 and 0.08 μM nocodazole for 6 h and then removed both. For prometaphase durations up to 48 min, none of the daughters arrested. However, for prometaphase durations 48–90 min, 24/32 (75%) of the daughters arrested (Figure 2B). For the progeny of mother cells that spent >90 min in prometaphase, all arrested. These results reveal that reduction in MCL-1 activity reduces the temporal tolerance for prolonged prometaphase.

This reduction in the temporal tolerance for prometaphase duration should not be due to residual MIM1, if any, in the daughter cells. First, continuous application of this concentration of MIM1 to control cells does not alter the cell cycle or produce a G1 arrest in any daughter cells. Second, continuous treatment with MIM1 starting at nocodazole washout does not alter the temporal tolerance for prolonged prometaphase. All daughters of mothers spending <1.5 h in prometaphase proliferated ($n = 32$) while all those born of mothers spending >1.5 h in prometaphase arrested ($n = 21$).

To back up our observations with MIM1 we knocked down MCL-1 and obtained similar results. Forty-eight hours after small interfering RNA (siRNA) transfection (knockdown efficacy shown in Supplemental Figure S1A), asynchronous cultures were treated with nocodazole for 6 h. Thirty-three of 74 (45%) daughters arrested though their mothers spent <1.5 h in prometaphase (Supplemental Figure S1C) a clear departure from the basic experiment (Figure 2A). Cells transfected with a control siRNA showed a similar response to prolonged prometaphase as untransfected cells (Figure 2A vs. Supplemental Figure S1D). Thus, reduced MCL-1 expression leads to a reduced temporal tolerance for prolonged prometaphase.

The gradual loss of MCL-1 during prolonged mitosis involves SCF^{Fbw7}-mediated degradation in some but not all cell types (Inuzuka *et al.*, 2011; Wertz *et al.*, 2011) (reviewed in Millman and Pagano [2011] and Sloss *et al.* [2016]). We next sought to slow the loss of MCL-1 during prolonged prometaphase by knocking down Fbw7. To test the practical effect of Fbw7 knockdown for prometaphase MCL1 levels in our RPE1 cells, asynchronous cultures were transfected with Fbw7 siRNA and 48 h later treated with 0.08 μM nocodazole for 3 h. Mitotic shake-off cells were then cultured for another 3 h in nocodazole so that all cells assayed spent 3–6 h in prometaphase. We also ran parallel cultures of untransfected cells for comparison. In two experiments, MCL1 levels in Fbw7 knockdown cells were 3.1 and 3.7 times higher than those of the untransfected cells (Supplemental Figure S1B), consistent with the results of Wertz *et al.* (2011) for the same siRNA sequence (Wei *et al.*, 2005).

We tested the effects of Fbw7 knockdown on the temporal tolerance for prolonged prometaphase. Forty-eight hours after siRNA transfection, asynchronous cultures were treated with nocodazole for 6 h. After drug washout, 15/58 (26%) of the daughters proliferated though their mothers spent 1.5–4.5 h in prometaphase (Figure 2C). The observation that not all daughters born of mothers spending >1.5 h in prometaphase proliferated may be due to cell-to-cell variability in Fbw7 knockdown in conjunction with both continued MCL1 synthesis during mitosis, balanced against its ubiquitin-independent proteolytic degradation (Sloss *et al.*, 2016). Although Fbw7 knockdown could alter the stability of other proteins, we did not observe any morphological or cell-cycle defects in daughter cells born of mothers spending <1.5 h in prometaphase. Additionally, FBW7 knockdown cells, not treated with nocodazole, repeatedly cycled with normal morphology and timing, indicating the lack of obvious pleiotropic effects (average 20 h, range 15–23 h, $n = 12$, vs. average of 20 h, range 16–26 h, $n = 40$, for untreated cells). Together,

these results indicate that partial stabilization of MCL1 levels prolongs the temporal tolerance for prolonged prometaphase.

Other antiapoptotic Bcl-2 family members

Although the expression levels of the antiapoptotic proteins Bcl-xL, Bcl-2, and Bcl-w do not change during prometaphase, their activity diminishes during prolonged prometaphase due to phosphorylation by Cdk1 (Terrano *et al.* [2010] and references therein). We next tested whether activity loss for these antiapoptotic Bcl family members during prolonged prometaphase participates in the G1 arrest of daughter cells. We did this because Bcl-xL is reported to be a primary negative regulator of apoptosis during mitotic arrest of some cancer cells (Shi *et al.*, 2011); its inhibition sensitizes cells to mitotic blockers (Bennett *et al.*, 2016); and Bcl-2/Bcl-xL inhibition potentiates apoptosis after prolonged mitosis in transformed cells (Colin *et al.*, 2015).

We treated asynchronous cultures with 1 μ M ABT-263 (Navitoclax) plus nocodazole for 6 h and then washed both out. ABT-263 inhibits Bcl-xL, Bcl-2, and Bcl-w (IC50s: <0.5 nM, <1 nM, and <1 nM, respectively; Vogler *et al.* [2009]). We found that all 41 daughters born of mothers spending <1.5 h in prometaphase proliferated (Supplemental Figure S2, top). Thus, inhibition of these other antiapoptotic Bcl-2 family members does not additionally predispose daughter cells to arrest for prometaphase durations <1.5 h, unlike the case for partial MCL-1 inhibition (Figure 2B). Thirty of 67 (45%) of the daughters of mothers spending >1.5 h in prometaphase arrested but remained alive (Supplemental Figure S2, top). Another three (asterisks) died at times ranging from 63–104 h after nocodazole removal. We also observed that 22 mother cells died during prometaphase (Supplemental Figure S2, bottom), consistent with the report that continuous application of 1 μ M ABT-263 promotes cancer cell killing during or just after slippage out of mitosis (Shi *et al.*, 2011). Surprisingly, 31 daughters still in the fields of view proliferated even though their mothers spent between 1.75 and 2.75 h in prometaphase. This is consistent with reports that MCL-1 can be translated during mitosis (Sloss *et al.*, 2016) and ABT-263 inhibition of Bcl-xL, Bcl-2, and Bcl-w can lead to increased expression and activity of MCL-1 (Williams *et al.*, 2017).

Effectors of apoptosis

To further test whether partial activation of the apoptotic pathway leads to daughter cell arrest, we tested whether diminishing proapoptotic elements of this pathway increases the temporal tolerance of cells for prolonged prometaphase. We treated asynchronous cultures with 2.5 μ M Bax channel blocker iMAC1 (IC50 520 nM) and nocodazole for 6 h and washed out both. Sixteen of 46 (35%) daughters proliferated, even though their mothers spent between 1.5 and 5 h in prometaphase (Figure 3A). The fact that not all daughters proliferated may reflect cell-to-cell variability in the loss of MCL-1, Bak activity, and incomplete inhibition of Bax channels.

To back up these observations we knocked down Bak and Bax with a mixture of validated siRNA constructs (Supplemental Figure S3A) and obtained similar results. Forty-eight hours after siRNA transfection, asynchronous cultures were treated with nocodazole for 6 h. We found that 9/42 (21%) daughters born from mothers spending 2–4 h in prometaphase proliferated. Together these results indicate that diminishing the apoptotic response increases the temporal tolerance for prolonged prometaphase.

To test the involvement of caspase activities in the G1 arrest of daughter cells after sufficiently prolonged prometaphase, we partially inhibited caspase activities during and after prolonged prometaphase with cell permeable z-VAD (OMe)-fmk, which is converted to the pan-caspase inhibitor z-VAD-fmk by intracellular

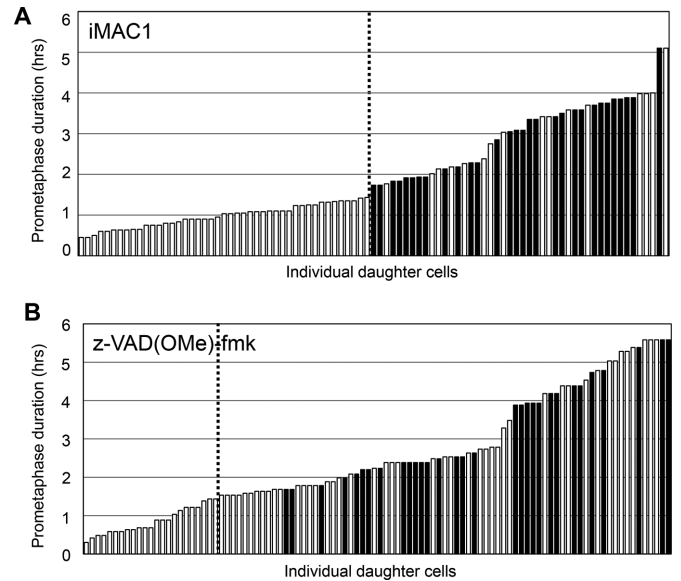


FIGURE 3: (A) Application of the Bax channel blocker iMAC1 just during prolonged prometaphase allows some daughter cells to proliferate even though their mothers spent between 1.5 and 5 h in prometaphase. Asynchronous cultures were treated with nocodazole plus 2.5 μ M iMAC1 for 6 h and both drugs washed out. For two pairs of daughter cells born of mothers spending >1.5 h in prometaphase, one daughter proliferated while its sister arrested. Significantly more daughters born of mothers spending >1.5 h in prometaphase proliferated relative to the basic experiment (Figure 2A): $p = 3.1 \times 10^{-6}$. For the daughters born of mothers spending <1.5 h in prometaphase, there was no significant decrease in the proportion of cells that proliferated ($p = 1.0$). (B) Pancaspase inhibitor allows some daughter cells to proliferate even though their mothers spent between 1.5 and 5.5 h in prometaphase. Asynchronous cultures were treated with 20 μ M z-VAD (OMe)-fmk for 12 h to allow drug entry/cleavage. After this agent was washed out, the cultures were treated with nocodazole for 6 h and daughter cells were continuously followed. Significantly more daughters born of mothers spending >1.5 h in prometaphase proliferated relative to the basic experiment (Figure 2A): $p = 3.2 \times 10^{-12}$. For the daughters born of mothers spending <1.5 h in prometaphase, there was no significant decrease in the proportion of cells that proliferated ($p = 1.0$).

esterase activity. We treated asynchronous cultures with 20 μ M z-VAD (OMe)-fmk for 12 h to allow drug entry/cleavage and after washout treated with nocodazole for 6 h. After nocodazole removal, 46/77 (60%) of the daughters remaining in the fields of view proliferated, even though their mothers spent 1.5–5.5 h in prometaphase (Figure 3B). The arrest of other daughters may be due to insufficient caspase inhibition in those particular cells. These results point to the functional importance of caspase activity in effecting the arrest of daughter cells after prolonged prometaphase.

To back up this functional approach, we assayed for the presence of activated caspase-3, an effector caspase, during prolonged prometaphase. This caspase is expressed as an inactive procaspase that is activated by proteolytic removal of the pro-domain and cleavage of the intersubunit linker (Riedl and Shi, 2004). We treated asynchronous cultures with nocodazole for 6 h and immediately fixed them for immunofluorescence labeling with a monoclonal antibody that recognizes only the large fragment of cleaved/activated caspase-3, not full-length caspase-3 (Supplemental Figure S3C). For comparison, we did the same for parallel cultures treated for 30 min with nocodazole. We then measured the fluorescence intensity in

individual mitotic cells in both populations. The distributions of individual mitotic cell gray-scale values for the two populations are shown in Supplemental Figure S3B. The mean values for the two populations are significantly different ($p < 0.00001$). These results reveal the presence of activated caspase-3 in mitotic cells and an increase in its levels during prolonged prometaphase.

Oxidative stress during prolonged prometaphase

Since oxidative stress can trigger apoptosis (reviewed in Wu *et al.* [2015] and Hampton and O'Connor [2016]) and lead to MCL-1 inactivation (Inoshita *et al.*, 2002), we tested whether oxidative stress participates during prolonged prometaphase in arresting daughter cells. We did this because oxygen levels under standard culture conditions (~20% or a pO_2 of 140 mm Hg at sea level) is higher than physiological conditions. In vivo mammalian levels range from 3% to 14% depending on the tissue (Sen and Roy, 2010).

To increase oxidative stress, asynchronous cultures were exposed to nocodazole and the oxidant $KBrO_3$ (1 mM) for 6 h and both washed out. Twenty-one of 30 (70%) of the daughters born of mothers spending <1.5 h in prometaphase arrested as did all daughters born of longer prometaphase durations; none later died (Figure 4A). This is not due to persistent oxidative damage because same preparation cells that were in interphase during the nocodazole/oxidant treatment cycled after $KBrO_3$ removal with the same timing (average 20.5 h, range 14–29 h, $n = 11$) as cells of separate control cultures (average 21 h, range 15–28 h, $n = 20$). Thus, increased oxidative stress reduced prometaphase durations that lead to daughter cell arrest.

We next used two means to determine whether reduced oxidative stress during mitosis would increase a cell's tolerance for prolonged prometaphase. First, we used external application of reduced glutathione, a tripeptide antioxidant normally present at 0.5–10 mM inside cells (Franco and Cidlowski, 2009). Asynchronous cultures were exposed to nocodazole and 50 μ M reduced glutathione for 6 h and both washed out. We observed the proliferation of 25/71 (35%) of the daughters born of mothers spending >1.5 h in prometaphase (Supplemental Figure S4A), a significant difference from the basic experiment (Figure 2A). Generally, cells do not import glutathione, but uptake has been reported in primary cultures of human RPE cells (Kannan *et al.*, 2001). In contrast, external application of 50 μ M oxidized glutathione did not promote the proliferation of daughters born of prometaphase durations >1.5 h (Supplemental Figure S4B). These results indicate that reduced oxidative stress can increase the temporal tolerance for prolonged prometaphase.

To further test whether reduced oxidative stress can influence the temporal tolerance for prolonged prometaphase, we worked with cells held at reduced oxygen levels. For control cells cultured at 3% oxygen, cell-cycle timing was normal (mitosis average 35 min, range 19–48 min, $n = 20$, and interphase 21 h, range 15–28 h, $n = 20$, values closely similar to those observed at atmospheric oxygen levels—mitosis average 35-min range, 18–57 min, $n = 20$, and interphase 20-h range, 16–26 h, $n = 20$).

Asynchronous cultures were treated with nocodazole for 10 h in medium equilibrated with 3% oxygen and then followed under atmospheric oxygen levels after drug washout. For daughters born of mothers spending 1.5–6.2 h in prometaphase, 14/35 (40%) proliferated while none proliferated for longer prometaphase durations (Figure 4B).

The arrest of daughter cells at low oxygen levels can occur without DNA damage. Even though 6-h prometaphase durations do not lead to DNA damage in our RPE1 cells (Uetake and Sluder, 2010), we tested for DNA damage in cells spending 6–10 h in prometaphase.

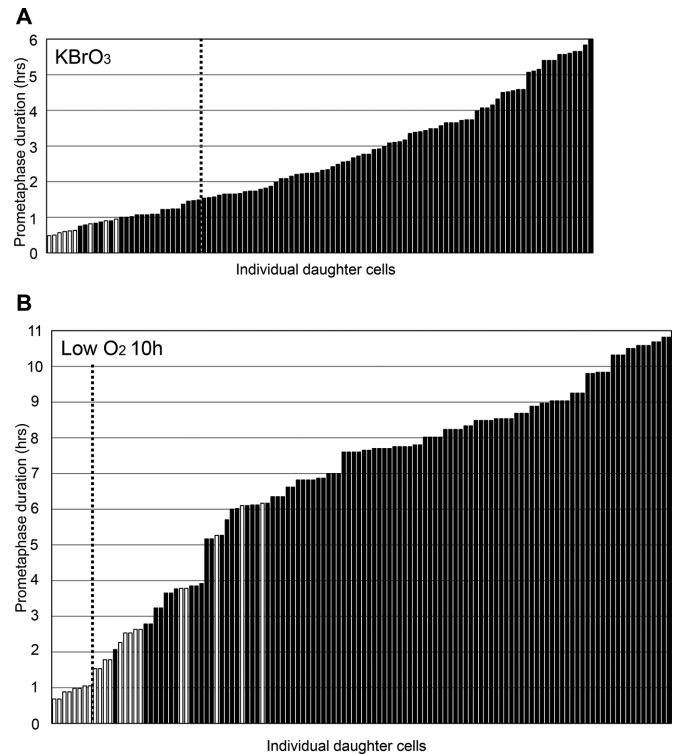


FIGURE 4: (A) Increased oxidative stress just during prometaphase reduces the temporal tolerance for prolonged prometaphase. Asynchronous cultures were treated with nocodazole plus $KBrO_3$ for 6 h and then both were washed out. Significantly fewer daughters born of mothers spending <1.5 h in prometaphase proliferated relative to the basic experiment (Figure 2A): $p = 4.8 \times 10^{-5}$. For the daughters born of mothers spending >1.5 h in prometaphase, there was no significant increase in the proportion of cells that proliferated ($p = 1.0$). (B) Reduced oxygen level increases the temporal tolerance for prolonged prometaphase up to 6 h but does not abrogate the G1 arrest of daughter cells born of longer prometaphase durations. Asynchronous populations were treated with nocodazole for 10 h in medium equilibrated with 3% oxygen. After nocodazole washout, the daughter cells were followed at ambient oxygen levels.

Immunofluorescence localization for H2AX foci in individual daughter cells previously followed in vivo revealed that 24/50 (48%) of the daughter cells born of mothers spending 6.8–10.7 h in prometaphase showed low levels of DNA damage that should contribute to their arrest (Supplemental Figure S4C). However, the other 26/50 (52%) of this group showed no DNA damage, yet they arrested. Together, these results indicate that reduced oxygen levels can increase the temporal tolerance for prolonged prometaphase up to ~6 h for some cells but does not prevent the arrest of cells held longer in prometaphase, even those that do not show DNA damage.

In separate experiments with up to 6-h prometaphase durations, we found that reduction of MCL1 activity at 3% oxygen shortened the temporal tolerance for prolonged prometaphase. As a baseline, we treated asynchronous cultures with nocodazole for 6 h at 3% oxygen and then followed daughter cells under atmospheric oxygen levels after drug removal. Consistent with the previous experiment, 34/52 (65%) of the daughter cells born of mothers held 1.5–6 h in prometaphase proliferated (Figure 5A). When the same experiment was conducted with 10 μ M MIM1 present just during the nocodazole treatment, all daughters of mothers spending >1.5 h in prometaphase arrested (Figure 5B). For mother cells that spent 1.2–1.5 h in prometaphase, 9/11 (82%) of the daughters arrested. Together

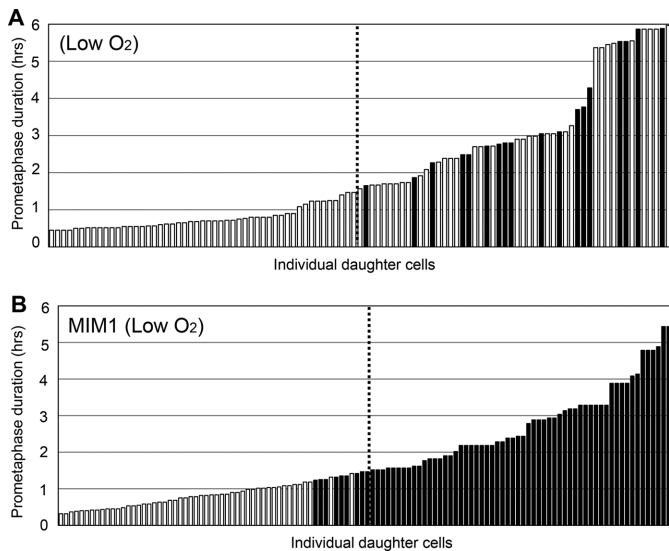


FIGURE 5: (A) Reduced oxygen increases the temporal tolerance for prometaphase prolonged up to 6 h. Asynchronous populations were treated with nocodazole for 6 h in medium equilibrated with 3% oxygen. After nocodazole removal, the daughter cells were followed at ambient oxygen levels. Significantly more daughters born of mothers spending >1.5 h in prometaphase proliferated relative to the basic experiment (Figure 2A): $p = 1.5 \times 10^{-14}$. For the daughters born of mothers spending <1.5 h in prometaphase, there was no significant decrease in the proportion of cells that proliferated ($p = 1.0$). (B) In contrast, partial inhibition of MCL1 activity at reduced oxygen level diminishes the temporal tolerance for prolonged prometaphase. Cells were treated with MIM1 plus nocodazole at ~3% oxygen for 6 h, and after washout of both drugs, daughter cells were continuously followed at ambient oxygen levels.

these results support the importance of MCL-1 activity loss in triggering a G1 arrest of daughter cells at physiological oxygen levels.

Post-mitotic activation of p38

The G1 arrest of daughters after prolonged prometaphase requires p38 activity after the mother cells complete mitosis. Inhibition of p38 activity just during prolonged prometaphase does not abrogate the G1 arrest of daughters while its continuous inhibition after nocodazole washout allows all daughters to proliferate regardless of the prometaphase duration for their mothers (Uetake and Sluder, 2010). To gain insight into these observations we determined when p38 is activated. We treated asynchronous cultures with nocodazole for 4 h and cultured shake-off mitotic cells for another 2 h in nocodazole. After drug removal, aliquots of shake-off cells were processed for western blotting at 0 min (prometaphase), 40 min (anaphase/telophase), 1.5 h (early G1), and 5 h (mid to late G1). Shake-off cells from control populations showed activating phosphorylations on Thr180 and Tyr182 of p38 (Figure 6, first lane), as previously reported for cells early in mitosis (Takenaka *et al.*, 1998; Campos *et al.*, 2002; Fan *et al.*, 2005; Tang *et al.*, 2008; Lee *et al.*, 2010). In contrast, after 2–6 h in prometaphase, activating phosphorylation levels in mitosis were low (0 min lane) and remained so through the end of mitosis (40 min lane). In early G1, activating phosphorylation levels markedly increased (1.5 h lane) and were low again by 5 h after drug removal (5 h lane). Such transient p38 activation is consistent with the report that continuous exposure to anisomycin leads to a ~1-h activation of p38 (Lu *et al.*, 2007). These results reveal that p38 activation occurs downstream of prolonged mitosis.

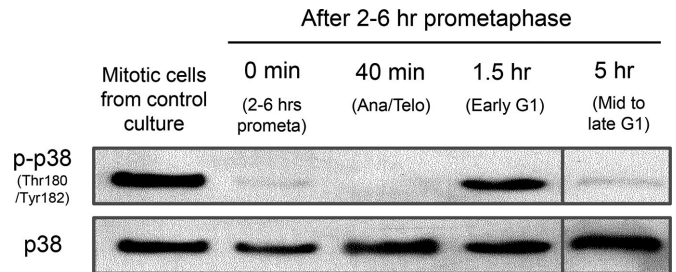


FIGURE 6: Transient post-mitotic activation of p38 in daughter cells born of mothers that spent 2–6 h in prometaphase. Western blots for activated p38, top row (phospho Thr180/Tyr182), and total p38, bottom row. First lane: mitotic shake-off cells from control culture. 0 min–5 h lanes: asynchronous cultures were treated with nocodazole for 4 h; mitotic cells were shaken off and cultured another 2 h in nocodazole before drug removal. Populations were prepared for blotting at the indicated times after nocodazole removal. 0 min lane: prometaphase cells collected just after drug removal. 40 min lane: late anaphase/telophase cells harvested 40 min after nocodazole removal. 1.5 h lane: G1 cells collected 1.5 h after nocodazole removal. 5 h lane: G1 cells harvested 5 h after nocodazole removal (from a different blot). The blots shown are representative of five experiments that showed identical results.

P38 activation after caspase inhibition

In this study, we earlier provided functional evidence that caspase activity participates in the G1 arrest of daughter cells after prolonged prometaphase. Given that p38 activity is required for daughter cell arrest after prolonged prometaphase, we tested whether caspase inhibition would influence the levels of activated p38 after prolonged mitosis. In three experiments, we treated asynchronous cultures with 20 μ M z-VAD (OMe)-fmk for 12 h to allow drug entry/cleavage. After inhibitor washout, the cells were treated with nocodazole for 4 h, and mitotic shake-off cells were kept in nocodazole for another 2 h. One and a half hours after nocodazole washout, the daughter cells were processed for Western blotting to quantify phospho-p38 levels. As a control, asynchronous cultures were treated same way with nocodazole but without 20 μ M z-VAD (OMe)-fmk pretreatment. For cultures treated with the caspase inhibitor, the levels of activated p38 were 42%, 64%, and 48% those of the parallel control cultures (example shown in Supplemental Figure S3D). These results reveal that caspase activity participates in the post-mitotic activation of p38. The fact that the levels of activated p38 were not zero in the caspase-inhibited cultures may be due to incomplete caspase inhibition in some cells, especially after the stress inherent in the shake off of their mothers.

Persistence of caspase activity

We previously found that when we prolonged prometaphase for >1.5 h and transiently inhibited p38 activity for at least 3 h just after mitosis to allow the daughters to get through G1, they progressed through interphase and divided again. Remarkably, all granddaughters arrested in G1, even though they were born from mitoses of normal duration (Uetake and Sluder, 2010). Thus, prolonged prometaphase can lead to an irreversible change in the properties of the daughter cells that cannot be “erased” by progression through interphase and normal mitosis.

To test whether persistent caspase activity participates in the granddaughter cell arrest, we first repeated the experiment. We treated asynchronous cultures with nocodazole for 6 h and, just after its removal, treated with the p38 inhibitor SB 203580 (10 μ M)

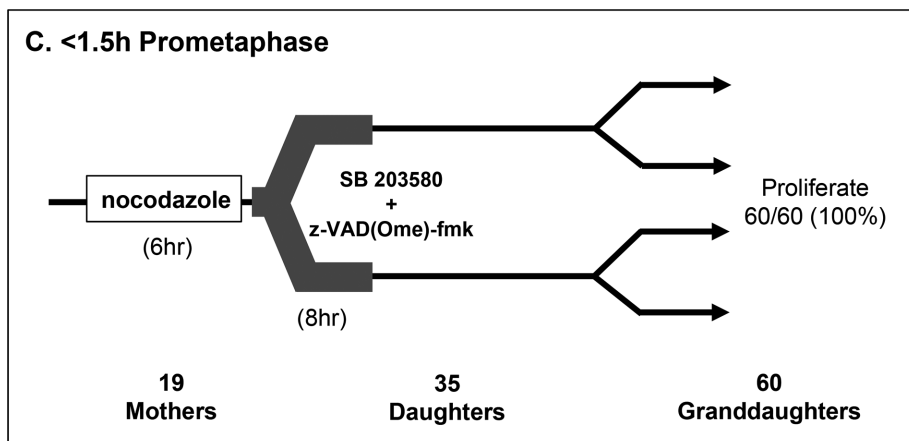
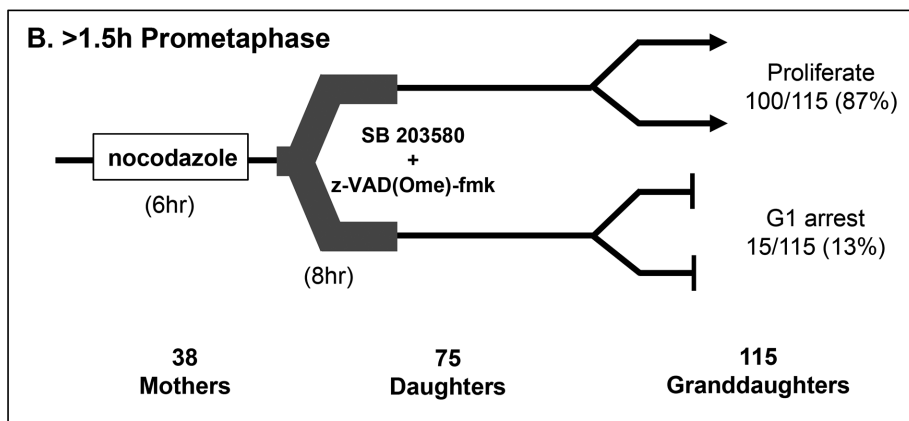
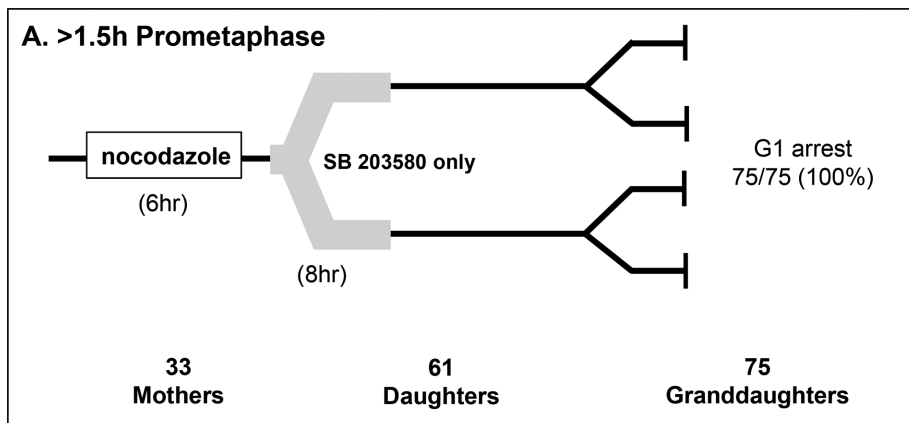


FIGURE 7: Persistent caspase activity leads to granddaughter cell arrest. (A) Asynchronous cultures were treated with nocodazole for 6 h and immediately after drug washout treated with the p38 inhibitor SB 203580 for 8 h. All daughters born of mothers spending >1.5 h in prometaphase progressed through interphase and divided again. However, all granddaughters still in the fields of view arrested. For the same preparation daughters born of mothers spending <1.5 h in prometaphase all granddaughters proliferated (not diagrammed). (B) The same experiment except that the pan-caspase inhibitor z-VAD(Ome)-fmk was included during the 8 h SB 203580 treatment. All daughter cells born of mothers spending >1.5 h in prometaphase progressed through interphase and divided again, and 87% of the granddaughters proliferated. (C) For the same preparation, daughters born of mothers spending <1.5 h in prometaphase all granddaughters proliferated.

for 8 h and then washed it out. All daughter cells born of mothers spending >1.5 h in prometaphase progressed through interphase and divided again. All granddaughters arrested, confirming our

For cells in the living mammal, those exposed to atmospheric oxygen, such as those of a healing surface wound, ~90 min in prometaphase may be the tipping point for a block to daughter

previous report (Uetake and Sluder 2010) (Figure 7A). We then did the same experiment but this time included the pan-caspase inhibitor z-VAD(Ome)-fmk (20 μM) along with the SB 203580 for 8 h. For mothers spending >1.5 h in prometaphase 100/115 (87%) of their granddaughters proliferated (Figure 7B). For the same preparation mothers spending <1.5 h in prometaphase, all 60 granddaughters proliferated (Figure 7C). These results provide functional evidence that sufficiently prolonged prometaphase in the mother cell leads to persistent caspase activity that later participates in granddaughter arrest.

DISCUSSION

Response to prolonged prometaphase

Moderate prolongation of prometaphase leads to the activation of the apoptosis pathway in the absence of DNA damage but not in an immediately lethal manner. This apoptotic response is slow for half of the daughter cells in which it runs to completion (death 33–288 h after mother cell mitosis), and for the other half it leads to senescence. Cells can commit to either apoptotic death or senescence—with apoptosis favored by stronger stresses (Childs *et al.*, 2014). Our observations are consistent with the report of sublethal activation of the apoptosis pathway during greatly prolonged prometaphase (Orth *et al.*, 2012) and the finding that cells can show a graded apoptotic response in which a small fraction of the mitochondria execute MOMP leading to sublethal levels of caspase activity (Ichim *et al.*, 2015).

Activation of the apoptotic pathway and daughter cell arrest involve the progressive loss of MCL-1 activity during prometaphase acting in concert with oxidative stress, which can promote MCL-1 inactivation (Inoshita *et al.*, 2002). Over time, these tip the balance of pro- and anti-apoptotic activities toward a consequential, albeit partial, apoptotic response. Though oxidative stress can participate, it is not needed. At 3% oxygen levels the temporal tolerance for prolonged prometaphase is increased up to 6 h for many cells but all daughters arrest after longer prometaphase durations. At such lowered oxygen levels, the extent of MCL-1 activity loss is still important, because when we diminish its activity during prometaphase, the temporal tolerance for prolonged prometaphase is reduced from 6 to ~1.2 h.

cell proliferation. For cells inside the body that experience ~3% oxygen levels the tolerance for prometaphase duration would be ~6 h. Cells of tissues, such as those in lung alveoli and the pulmonary aorta, which normally experience up to 14% oxygen, may have an intermediate tolerance for prolonged prometaphase.

Although the expression levels of the antiapoptotic proteins Bcl-xL, Bcl-2, and Bcl-w do not change during prometaphase, their activity diminishes due to phosphorylation by Cdk1 (Terrano *et al.*, 2010). Unlike partial MCL-1 inhibition, diminishment of their activities did not promote the arrest of daughters born of mothers spending <1.5 h in prometaphase. Thus, for RPE1 cells MCL1 activity loss during prolonged prometaphase more directly promotes daughter cell arrest than activity loss for these other antiapoptotic Bcl-2 family members.

In our initial study (Uetake and Sluder, 2010), we prolonged prometaphase with the proteasome inhibitor MG132 at atmospheric oxygen levels and found that all daughter cells arrested if born of mothers spending >1.5 h in prometaphase. This seems paradoxical because MG132, while present, should protect MCL-1 from proteolytic degradation, thereby allowing daughter cell proliferation regardless of prometaphase duration. An explanation for this daughter cell arrest is that mother cells spending longer in prometaphase suffer more accumulated oxidative stress. MG132 is said to induce apoptosis through the formation of reactive oxygen species (Wu *et al.*, 2002; Han and Park, 2010; Guo and Peng, 2013). Also, Inoshita *et al.* (2002) report that oxidative stress induces c-Jun N-terminal kinase (JNK) activation that leads to the phosphorylation and inactivation of MCL-1. This in turn would promote daughter cell arrest despite inhibition of the proteolytic loss of MCL-1.

When prometaphase is prolonged with nocodazole or monastrol at atmospheric oxygen levels, there is a sharp break between prometaphase durations that do and do not allow daughter cell proliferation (Uetake and Sluder, 2010). For all other experimental conditions, there is a range of prometaphase durations over which some daughters arrest and some proliferate. During prolonged prometaphase, should there be any cell-to-cell variation in the changing balance of pro- and anti-apoptotic activities when cells are at the cusp of activating a consequential apoptotic response (see Flusberg and Sorger [2015]), the lack of a sharp break between daughter cell proliferation and arrest is not surprising. Under less than extreme circumstances apoptosis is not a digital live or die phenomenon; it can be partial (Orth *et al.*, 2012; Ichim *et al.*, 2015) and even be reversible (Tang *et al.*, 2012). This may explain why partially stabilizing MCL-1 levels or partially inhibiting proapoptotic factors such as BAX or caspases allows some but not all of the daughters to proliferate after their mothers spent >1.5 h in prometaphase. In this regard, we noted in some experiments a few cases in which one daughter arrested while its sister proliferated after prolonged prometaphase. Since Mcl-1 activity participates in the intrinsic (mitochondrion based) apoptotic pathway, this phenomenon could be due to unequal inheritance of the small percentage of mitochondria undergoing MOMP in response to sublethal stress (Ichim *et al.*, 2015).

The p53 stabilizing activities of 53BP1 and USP28 are required for daughter cell arrest downstream from prolonged prometaphase (Fong *et al.*, 2016; Lambrus *et al.*, 2016; Meitinger *et al.*, 2016). How the p53-53BP1-USP28 complex is assembled and initiates p53 stabilization after prolonged mitosis, in the absence of DNA damage, is an intriguing issue that has yet to be investigated (reviewed in Lambrus and Holland [2017]). P53 stabilization does not appear to be due to activity of this complex during prometaphase, because we did not find an increase in p53 levels for cells held in prometaphase for almost 4 h (Uetake and Sluder, 2010). Moreover, at low

oxygen levels, prometaphase durations up to 6 h allowed many daughter cells proliferate, suggesting no significant prometaphase accumulation of p53.

The prometaphase loss of 53BP1 from kinetochores (in ~88% of the cells by 60 min into mitosis) raised the issue that this delocalization could be how cells sense prometaphase duration and trigger a block of daughter cell proliferation (Fong *et al.*, 2016). However, under low-oxygen conditions we find that prometaphase durations up to 6 h allow many daughter cells to proliferate. Thus, daughter cells can proliferate when born from mitoses in which 53BP1 should be gone from all kinetochores well before anaphase.

For cells synchronized in G2 and then released into mitosis, prolonging prometaphase with the Eg5 inhibitor monastrol can lead to centrosome fragmentation and mother–daughter centriole separation (Karki *et al.*, 2017). We also observed centrosome expansion during prolonged prometaphase in the presence of monastrol. However, we did not observe centriole separation or spindle multipolarity when prometaphase was prolonged up to 6 h with nocodazole.

Apoptosis and daughter cell arrest

An important question is how activation of the apoptosis pathway during prolonged prometaphase leads to the G1 arrest of the daughter cells. This arrest is dependent on p38 activity after mitosis and the subsequent activity of the p53 (Uetake and Sluder, 2010; Fong *et al.*, 2016; Lambrus *et al.*, 2016; Meitinger *et al.*, 2016). Since p38 activates p53 (Cuenda and Rousseau, 2007), the question is how the apoptotic response during prometaphase activates p38. This occurs by caspase cleavage of MEKK-1, which produces a kinase active C-terminal fragment that activates MKK3 and MKK6 that in turn phosphorylate the p38 proteins at activating sites (Cardone *et al.*, 1997; Nakagami *et al.*, 2001; Zarubin and Han, 2005; Cuenda and Rousseau, 2007). P38 activates p53 after mitosis and 53BP1/USP28 activities stabilize p53 to allow its accumulation (Fong *et al.*, 2016; Lambrus *et al.*, 2016; Meitinger *et al.*, 2016). P53 activation leads to p21 expression that arrests the cell cycle in G1.

Activation of p38 downstream of the apoptotic pathway (Nakagami *et al.*, 2001) is supported by our finding that p38 does not show activating phosphorylations at the end of prolonged prometaphase; rather it is transiently phosphorylated ~1.5 h after nocodazole removal. Also, inhibition of p38 activity just during prolonged prometaphase does not abrogate the G1 arrest of daughter cells while its continuous inhibition after prolonged mitosis allows all daughters to proliferate regardless of prometaphase duration for their mothers (Uetake and Sluder, 2010).

The importance of caspase activity for the G1 arrest of daughter cells after prolonged prometaphase is evident from our finding that caspase inhibition during and after prolonged prometaphase allows over half of the daughters to proliferate even though their mothers spent between 1.5 and 5.5 h in prometaphase. This is in contrast to no daughter cell proliferation in the absence of caspase inhibition after >1.5 h prometaphase for the mothers. We also found that caspase inhibition reduced the levels of p38 activation after prolonged prometaphase.

For the daughters of mother cells that spent >1.5 h in prometaphase, transient inhibition of p38 activity just after mother-cell mitosis allows them to progress through interphase and a subsequent mitosis of normal duration. Remarkably, all granddaughters arrest suggesting an irreversible change in the properties of the progeny of such mother cells (Uetake and Sluder [2010] and present study). Here we provide functional evidence that this change involves persistent caspase activity in the daughter cells. When we inhibited

caspase activity starting at the transient block to p38 activity after prolonged mitosis of the mother, the great majority of the granddaughters proliferated without additional inhibition of p38.

Backing up the mitotic checkpoint

The human cell appears to have layers of protection against mitotic defects that can lead to genomic instability and the consequent risk of transformation. The mitotic checkpoint will hold cells in mitosis for hours until defects in bipolar chromosome attachment are resolved. If prometaphase is grossly prolonged, the sustained activation of the apoptotic pathway leads to DNA damage and its consequent p53 response that prevents daughter-cell proliferation. However, environmental perturbations can lead to unresolved mitotic defects that prolong prometaphase but eventually allow for mitotic checkpoint satisfaction and completion of an error prone mitosis (Brito *et al.*, 2008). In real life, such defects could arise from environmental toxins, heat, radiation, or oxidative stress in tissues that experience higher oxygen levels. Our present results reveal that partial activation of the apoptotic pathway protects the cell from mitotic defects that modestly prolong prometaphase yet allow the satisfaction of the mitotic checkpoint before DNA damage occurs.

MATERIALS AND METHODS

Cell culture and reagents

hTERT-RPE1 cells were cultured as described previously (Uetake and Sluder, 2004). Nocodazole (#M1404), L-glutathione reduced (#G4251), L-glutathione oxidized (#G4376), and KBrO₃ (#309087) were purchased from Sigma-Aldrich. MCL-1 inhibitor MIM1 (#444130), Bax channel blocker (iMAC1, #196805), SB 203580 (#559389), and pan-caspase inhibitor (z-VAD(OMe)-fmk, #627609) were purchased from EMD Millipore. ABT-263 (Navitoclax) was purchased from ApexBio. Annexin V-FITC early apoptosis detection kit (#6592) was purchased from Cell Signaling and used according to the manufacturer's instructions. Senescence-specific β -galactosidase staining was performed according to Dimri *et al.* (1995) with reagents purchased from Sigma-Aldrich. Anti-p38 (#9218), anti-phospho-p38 (#9211; Thr180/Tyr182), anti-MCL-1 (#4572), anti-cleaved caspase-3 (#9664; Asp175), and anti- α -tubulin (#3873) antibodies were obtained from Cell Signaling. Western blotting was performed as described previously (Uetake and Sluder, 2010). Cells stained for β -galactosidase were observed with phase contrast optics and color images recorded with a Canon Rebel XS DSLR camera and EOS Utility software (Canon). The images were processed with Adobe Photoshop to enhance the blue color of the β -galactosidase reaction product; all images were processed identically and full frame. Immunostaining of cleaved caspase-3 was performed according to the protocol provided by the manufacturer. The cells were observed on a Leica DMI 6000B using a 20 \times /0.40 objective, and images were taken with a Leica DFC365 FX camera. Fluorescence intensity of Alexa fluor 488 was measured using Leica Rebel XS software.

RNA interference

Certified siRNAs against MCL-1 (SignalSilence Mcl-1 siRNA I, #6315), Bak (SignalSilence Bak siRNA I, #6486), and Bax (SignalSilence Bax siRNA I, #6321) were purchased from Cell Signaling. All SignalSilence siRNA products from Cell Signaling Technology were tested by the manufacturer and shown by the company's Western blot analysis to efficiently knock down the target proteins. Annealed siRNA against FBW7 (same sequence as in Wertz *et al.* [2011] and Wei *et al.* [2005]), and control siRNA (siGENOME Non-targeting siRNA#1) were obtained from GE Dharmacon. All siRNAs were di-

luted to 20 μ M with nuclease-free water as a stock solution. Two microliters of stock solution was mixed with 5 μ l of Lipofectamine (Invitrogen) and then introduced onto the cells for 2 h; the cells were then washed with culture medium and cultured for 48 h (Uetake and Sluder, 2010).

Prolonging prometaphase

Asynchronous cultures were mounted in observation chambers (Uetake and Sluder, 2012) for 6 h in the presence of 0.08 μ M nocodazole. Fields were marked with a diamond scribe and continuously followed by video time-lapse microscopy to record when individuals entered mitosis. Cells entering mitosis assembled short bipolar spindles or monopolar spindles, both with monooriented chromosomes that prevented mitotic checkpoint satisfaction. None slipped into interphase in 6 h. Cells entering mitosis early spent several hours in prometaphase before drug washout while cells entering mitosis shortly before drug removal spent little extra time in prometaphase. After 6 h, the bottom of the observation chamber was removed, the drug washed out with fresh medium several times, and the chamber reassembled with fresh medium for further observation of the same fields for up to 100 h. In this way, we knew precisely the prometaphase duration for each mother cell—here defined as mitosis entry to anaphase onset—and the fate of her daughters. All experiments involved multiple preparations and time-lapse runs conducted on separate days. During the film runs, the medium was changed every 2 d. Live-cell imaging was conducted with Leica DMRXE microscopes equipped with phase-contrast optics (10 \times , 0.3 NA objectives). Images were taken with Orca ER, Orca 100 (Hamamatsu), and RetigaEXi (QImaging Corp.) cameras, acquired with HCLImage software (Hamamatsu), and exported as AVI movies.

Reduced oxygen levels

Culture medium was equilibrated with an atmosphere of 3% oxygen, 5% CO₂, 92% nitrogen overnight in a sealed enclosure (Modular Incubator Chamber from Billups-Rothenberg) in the incubator. Observation chambers were filled with the equilibrated media and then sealed with a cell bearing coverslip within 30 s under atmospheric conditions. At the end of 6 or 10 h, depending on the experiment, the drugs were washed out with medium that was equilibrated with atmospheric oxygen level.

Statistical analysis

Statistical tests were performed using "R" Core Team (RC Team, 2016). The significance of differences in the proliferative capacity of daughter cells between the basic experiment (Figure 2A) and other experimental conditions (Figures 2, B and C, 3, A and B, 4A, and 5A and Supplemental Figures S1, C and D, S3A, and S4, A and B) was assessed using pairwise Fisher's exact tests on the 2 \times 2 contingency tables of counts of proliferating versus nonproliferating daughter cells, separately in two bins based on prometaphase duration: a one-tailed test in the " ≤ 90 min" bin was used to test for a decreased proportion of proliferating cells compared with the basic experiment (in which 52 of 57 daughter cells were proliferating), and a one-tailed test in the ">90 min" bin was used to test for an increased proportion of proliferating cells compared with the basic experiment (in which 0 of 54 daughter cells were proliferating). Even within these bins, if the probability of nonproliferation increases as the prometaphase duration increases, then differences in the distributions of durations between conditions can bias these statistical tests. Thus, we also performed analogous Fisher's exact tests on subsets of cells selected to counter this bias, as detailed below;

all differences that were significant at $p < 0.01$ remained so when using the more conservative tests, and it is these more conservative p values.

Because requiring distributions of durations to be nearly the same between conditions can result in many cells being excluded, we allowed the distributions to differ, but only in a direction that biases the one-tailed tests to be more conservative. For the “ ≤ 90 minute” bin, in which we tested for a decreased proportion of proliferating cells compared with the basic experiment, we allowed the distribution to be skewed toward shorter durations than for the basic experiment. In this case, we used the Hungarian method (function `solve_LSAP` in the R package `clue`; Hornik [2005]) to find a maximum-sized pairing of cells between experiments subject to the constraint that each cell in the basic experiment can only be paired with a cell having shorter-or-equal prometaphase duration. Viewing the pairing as a matching in a bipartite graph with cells as vertices, we assigned weight 0 to illegal edges (those that violate the constraint) and weight $1 + \alpha e^{-\delta}$ to other edges, where δ is the difference in prometaphase durations (which gives a preference to pairings between cells with more-similar durations) and $\alpha = 1/(N + 1)$, where N is the lesser of the cell counts for the two experiments being compared; this α is small enough to ensure that the maximum-weight matching also has the maximum cardinality of legal edges, so that as few cells as possible are excluded. The maximum-weight matching need not be unique, so to avoid bias the algorithm was run without regard to proliferation status and with cell order randomized. For the “ > 90 minute” bin, in which we tested for an increased proportion of proliferating cells compared with the basic experiment, we allowed the distribution to be skewed toward longer durations than for the basic experiment, using an analogous procedure.

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