"Wait anaphase" signals are not confined to the mitotic spindle

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ABSTRACT **The spindle assembly checkpoint ensures the faithful inheritance of chromosomes by arresting mitotic progression in the presence of kinetochores that are not attached to spindle microtubules. This is achieved through inhibition of the anaphase-promoting complex/cyclosome by a kinetochore-derived "wait anaphase" signal known as the mitotic checkpoint complex. It remains unclear whether the localization and activity of these inhibitory complexes are restricted to the mitotic spindle compartment or are diffusible throughout the cytoplasm. Here we report that "wait anaphase" signals are indeed able to diffuse outside the confines of the mitotic spindle compartment. Using a cell fusion approach to generate multinucleate cells, we investigate the effects of checkpoint signals derived from one spindle compartment on a neighboring spindle compartment. We find that spindle compartments in close proximity wait for one another to align all chromosomes before entering anaphase synchronously. Synchrony is disrupted in cells with increased interspindle distances and cellular constrictions between spindle compartments. In addition, when mitotic cells are fused with interphase cells, "wait anaphase" signals are diluted, resulting in premature mitotic exit. Overall our studies reveal that anaphase inhibitors are diffusible and active outside the confines of the mitotic spindle from which they are derived.**

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INTRODUCTION

Accurate chromosome inheritance during cell division is necessary for the development and maintenance of all organisms. Failure to properly segregate genetic material results in the generation of aneuploid cells (cells with too few or too many chromosomes), an event associated with disease states such as infertility and cancer (Santaguida and Amon, 2015). Thus cells have developed an elegant surveillance system called the spindle assembly checkpoint (SAC), which suspends the initiation of anaphase—the separation of chromatids toward opposite cell poles—until all the chromosomes are positioned to be equally inherited (Musacchio, 2015). The SAC monitors attachment of spindle microtubules (MTs) to large protein complexes called kinetochores (KTs), which reside at the centromere of each chromosome (Kops and Shah, 2012). Unbound KTs generate a molecular signal that ultimately manifests in the cell-wide inhibition of anaphase onset (Rieder *et al.*, 1995). This "wait anaphase" signal, which is composed of a multimeric protein complex called the mitotic checkpoint complex (MCC), is generated when Mad1 induces a conformational change in Mad2 from an open to a closed state (Luo *et al.*, 2004; Mapelli and Musacchio, 2007; Faesen *et al.*, 2017; Ji *et al.*, 2017). It is on the scaffold of a closed Mad2 molecule that the MCC—which consists of Mad2, Bub3, BubR1, and Cdc20—is assembled (Sudakin *et al.*, 2001; Han *et al.*, 2013). The MCC delays anaphase onset by inhibiting the activity of the anaphase-promoting complex/cyclosome (APC/C)—the aptly named E3-ubiquitin ligase whose action is required for progression into anaphase—through sequestration of the APC/C-activating subunit Cdc20 (Yamaguchi *et al.*, 2016). Inhibition of the APC/C prevents the targeting and subsequent degradation of key mitosis-promoting factors, including cyclin B and securin (Musacchio, 2015). This checkpoint is exceptionally robust. Studies have shown that the inhibitory "wait anaphase" signals generated by even a single unbound KT can arrest a cell in mitosis (Rieder *et al.*, 1995; Collin *et al.*, 2013).

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Abbreviations used: A, anaphase; APC/C, anaphase-promoting complex/cyclosome; BPS, bipolar spindle; I, interphase; KT, kinetochore; M, metaphase; MCC, mitotic checkpoint complex; MT, microtubule; NEBD, nuclear envelope breakdown; PCC, premature chromosome condensation; PM, prometaphase; SAC, spindle assembly checkpoint.

Nearly three decades of intensive research have led to the characterization of the molecular mechanisms by which the SAC senses and responds to chromosome-attachment defects. However, we still do not fully understand how inhibitory MCC complexes generated from a single KT can influence the activities of the entire cell. This mechanism no doubt relies on the diffusible properties of KT-derived inhibitory signals.

Rieder *et al.* (1997) observed mitosis in fused mammalian cells possessing two spindle compartments, defined as an assembled spindle and the associated molecular components that originate from a single nucleus. They made two key observations: 1) the unattached KTs in one spindle compartment did not cause a mitotic checkpoint arrest in the neighboring spindle compartment, and 2) when one spindle compartment initiated anaphase, the neighboring spindle compartment also initiated anaphase, regardless of the alignment status of its own chromosomes. These observations prompted them to conclude that the activity and diffusibility of "wait anaphase" signals (i.e., active MCC complexes) were restricted to the spindle from which they were generated, but "go anaphase" signals were global and dominant. The notion that MCC molecules are spindle restricted has remained a dominant model. Evidence supporting the MCC restriction model includes the discovery of the spindle matrix: a proteinaceous fusiform structure that embodies the mitotic spindle (De Souza *et al.*, 2009; Lince-Faria *et al.*, 2009; Schindelin *et al.*, 2012; Yao *et al.*, 2012; Schweizer *et al.*, 2015). Work using *Drosophila* and human cells has shown that Mad1 and Mad2 remain enriched within this structure, suggesting that indeed, components of the SAC and MCC may be restricted in their diffusion away from the spindle compartment (Lince-Faria *et al.*, 2009; Yao *et al.*, 2012; Schweizer *et al.*, 2015).

Characterization of the molecular mechanisms underlying SAC signaling has raised the possibility that MCC complexes may not be confined to the spindle compartment and may in fact diffuse throughout the cytoplasm of a mitotic cell (Yu, 2002; Cleveland *et al.*, 2003; Hardwick, 2005; Ciliberto and Shah, 2009; Vanoosthuyse and Hardwick, 2009; Lara-Gonzalez *et al.*, 2012; Faesen *et al.*, 2017; Ji *et al.*, 2017). For example, an important prediction of the spindle restriction model is that the target of the MCC—the APC/C—should also be restricted to the spindle compartment such that it can be sufficiently inhibited by the MCC to prevent anaphase onset. If the APC/C^{CDC20} pool was not spindle restricted but MCC complexes were, then the non–spindle-restricted pool of APC/C^{CDC20} would be active and would thus promote anaphase onset irrespective of KT–MT attachment status. However, in mitotic cells, subcellular pools of the APC/C are found both within the spindle compartment (e.g., spindle poles, kinetochores, chromosomes) and throughout the cytoplasm, suggesting that there may be a cytoplasmic inhibitor of the APC/C (Topper *et al.*, 2002; Acquaviva *et al.*, 2004; Torres *et al.*, 2010; Sivakumar *et al.*, 2014; Sivakumar and Gorbsky, 2015).

Here we demonstrate that "wait anaphase" signals are not restricted to the mitotic spindle compartment from which they originate and are in fact diffusible. Similar to the cell fusion experiments conducted by Rieder *et al.* (1997), we fused mitotic cells and examined the behavior of spindle compartments that share a common cytoplasm. We set out to test three predictions of how "wait anaphase" signals should behave if they are restricted to the confines of the mitotic spindle. First, spindle compartment–restricted "wait anaphase" signals should be unable to influence the behavior and mitotic progression of other spindles within a shared cytoplasm. Second, spindle compartment–restricted "wait anaphase" signals should be insensitive to cellular diffusion barriers. Finally, inhibitory activities of spindle compartment–restricted "wait anaphase" signals should not be affected by cytoplasmic dilution. In contrast to earlier results, we find that mitotic spindles within close proximity wait for one another to align their chromosomes before initiating anaphase in synchrony. In contrast, spindles that remain far apart or are separated by a cellular constriction do not undergo synchronous anaphase. We also find that when mitotic cells are fused with interphase cells, preexisting mitotic spindle compartments exit mitosis prematurely, suggesting that the "wait anaphase" signals become diluted by nonmitotic cytoplasm. These observations support a model in which KT-derived "wait anaphase" signals can diffuse away from the source spindle compartment and into the cytoplasm to levels that are sufficient to prevent anaphase onset. Our findings provide new insight into the molecular mechanisms governing the activity of the spindle assembly checkpoint.

RESULTS

Synchronized and fused PtK1 cells exhibit normal mitotic timing

To enrich for mitotic PtK1 cells for use in our fusion experiments, we treated cells with the CDK1 inhibitor RO3306 to arrest cells at the G2/M boundary (Vassilev *et al.*, 2006). We found that ~30% of cells entered mitosis within 1 h after washout from a 20-h treatment with RO3306 (Figure 1A). We compared the timing of mitotic progression in asynchronous cells, synchronized cells, and synchronized cells exposed to fusion medium (but those that remained unfused) and found that treatment with RO3306 or fusion medium did not significantly affect the duration of prometaphase (defined as the time from nuclear envelope breakdown [NEBD] to chromosome alignment) or metaphase (defined as the time from chromosome alignment to chromatid separation; Figure 1, B and C). Using partially synchronized populations of PtK1 cells, we generated fused cells possessing multiple spindle compartments within a single shared cytoplasm (see *Materials and Methods*).

Spindles wait for one another to initiate anaphase

From their study of fused mitotic cells, Rieder *et al.* (1997) concluded that "wait anaphase" signals were restricted to the spindle compartment from which they were generated. They based this conclusion on the observation that in bi-spindled cells, unbound kinetochores in one spindle compartment did not delay anaphase onset in the neighboring compartment. However, the observed behaviors could also be explained if 1) the inhibitory MCC complexes were diffusible, but 2) the spindle compartments were too far away from one another for the MCC complexes generated from one compartment to impose a mitotic arrest on the neighboring compartment. If this were the case, we would predict that spindle compartments in sufficiently close proximity would exchange/share "wait anaphase" signals, and only when the SAC of both compartments were satisfied would synchronous anaphase ensue (Figure 2A, left).

To determine whether spindle compartments share "wait anaphase" signals, we imaged fused mitotic cells over time and scored the frequency of synchronous anaphase onset. Strikingly, time-lapse imaging revealed numerous fused bi-spindled cells in which the first spindle to reach metaphase (the "early" spindle) appeared to delay anaphase onset until the other spindle (the "late" spindle) aligned all of its chromosomes. Soon after this occurred, both spindle compartments synchronously entered anaphase (Figure 2B). In these cases, the early spindle delayed anaphase onset for up to three times longer (maximum, 30 min; average, 21.2 min) than the mean duration of metaphase in

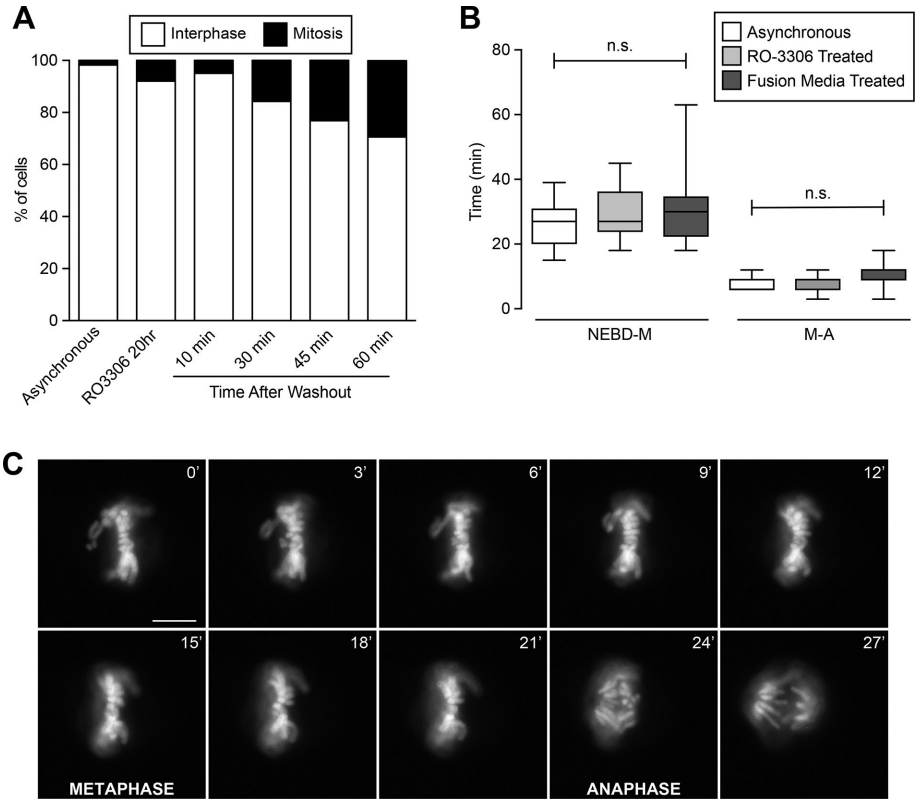

FIGURE 1: The CDK1 inhibitor RO3306 partially synchronizes PtK1 cells in mitosis without affecting mitotic timing. (A) Fraction of cells in the indicated phase of the cell cycle in the absence or presence of RO3306 followed by drug washout, as indicated. A 20-h RO3306 treatment followed by drug washout leads to a partial enrichment of cells in mitosis. (B) Duration of prometaphase (NEBD to metaphase [NEBD-M]) and metaphase (metaphase to anaphase [M-A]) in asynchronous cells, RO3306 synchronized cells, or RO3306 synchronized cells exposed to fusion medium (specifically, those that remain unfused; see *Materials and Methods*). Significance was determined using one-way analysis of variance tests (n.s., not significant). (C) Example time frames depicting mitotic progression (starting at late prometaphase) of a PtK1 cell stably expressing H2B-GFP treated with RO3306 for 20 h and then released into non–RO3306-containing medium. Scale bar, 10 μm.

nonfused cells (average, 9.4 min; Figures 1B and 2C, "Single Spindle"). Of interest, once the late spindle completed aligning its chromosomes, it progressed into anaphase more quickly than control spindles (5.7 vs. 9.4 min; $p < 0.001$; Figure 2C). This supports the notion that cells become primed to exit mitosis as they accumulate KT attachments to spindle microtubules. Previous studies indicate that this priming is due to a combination of 1) decreased formation of new inhibitory MCC complexes and 2) active disassembly of existing MCC complexes (Ciliberto and Shah, 2009; Collin *et al.*, 2013).

It should be noted that synchronous anaphase between two spindles was observed in the seminal study using fused cells to study checkpoint activity conducted by Rieder *et al.* (1997). However, they did not include these cells in their analysis due to the high variability of metaphase duration that they had calculated previously for PtK1 cells. Specifically, they reported that the time spent in metaphase varied from 9 to 49 min (Rieder *et al.*, 1997). Because of this high variability in metaphase duration, it could not be determined whether one spindle was delayed in anaphase onset due to its neighbor spindle or, alternatively, the mitotic timing of the "delayed" spindle fell into the reported normal range. In our studies, we found that the duration of metaphase in PtK1 cells was shorter (9.4 min) and that the range was narrower (3–18 min) than reported in previous studies. Thus we are confident that the behavior resulting in synchronous anaphase is not due to variability of mitotic timing but instead to cell-wide inhibition of anaphase onset imparted by the spindle containing unbound KTs. In addition, Rieder *et al.* (1997) included fused cells possessing monopolar or multipolar spindles in their analysis. Owing to the complex signaling and mitotic timing that occurs in such abnormal spindles, we excluded cells containing multipolar or monopolar spindles from our analysis.

Asynchronous anaphase occurs when "wait anaphase" signals are subject to diffusion barriers

To obtain a larger sample size than was feasible with our live-cell experiments, we implemented a fixed-cell protocol to analyze mitotic cell fusion events. For these experiments, we first fused synchronized PtK1 cells cultured on coverslips as described earlier. After exposure to fusion media, we returned the cells to normal growth conditions for 1 h to allow for progression into mitosis and then subjected them to fixation and immunostaining. We determined the stage of mitotic progression in each spindle as follows: 1) using differential interference contrast microscopy, we assessed whether sister chromatids were attached or separated, as anaphase chromatids become separated from one another and move toward opposite spindle poles; 2) we used immunofluorescence to visualize the mitotic spindle, as anaphase spindles contain short KT fibers near the poles and long interpolar MTs (McIntosh, 2016); and 3) we used immunofluorescence to detect the SAC protein Mad1, which localizes to unattached but not attached KTs (Figure 3B, merged insets; Howell *et al.*, 2004).

As before, we predicted that if "wait anaphase" signals were diffusible, then one spindle with an unattached kinetochore should generate sufficient signal to keep cytoplasmic (i.e., diffusive) MCC levels above the threshold needed to keep both spindles in mitosis (Figure 3Ai, top). Alternatively, if "wait anaphase" signals were spindle restricted, then a shared cytoplasmic volume would have no effect on anaphase onset of each spindle (Figure 3Ai, bottom). Using our fixed-cell approach, we observed many cells (44 of 55) in which two mitotic spindles appeared to be undergoing synchronous anaphase (Figure 3B, left). Of interest, and in stark contrast, we also observed cells in which only one of the two spindles was undergoing anaphase, indicating asynchrony of anaphase onset (11 of 55; Figure 3B, right). We noticed that the spindles within these asynchronous anaphase cells were at greater distances from one another than the spindles in cells that underwent synchronous anaphase (Figure 3B; compare "Synchronous" to "Asynchronous"). In addition, cells that underwent asynchronous anaphase typically formed a cytoplasmic constriction point between the two spindles (Figure 3, B, right, and D). If "wait anaphase" signals are confined to the spindle compartment, these signals should be insensitive to diffusion

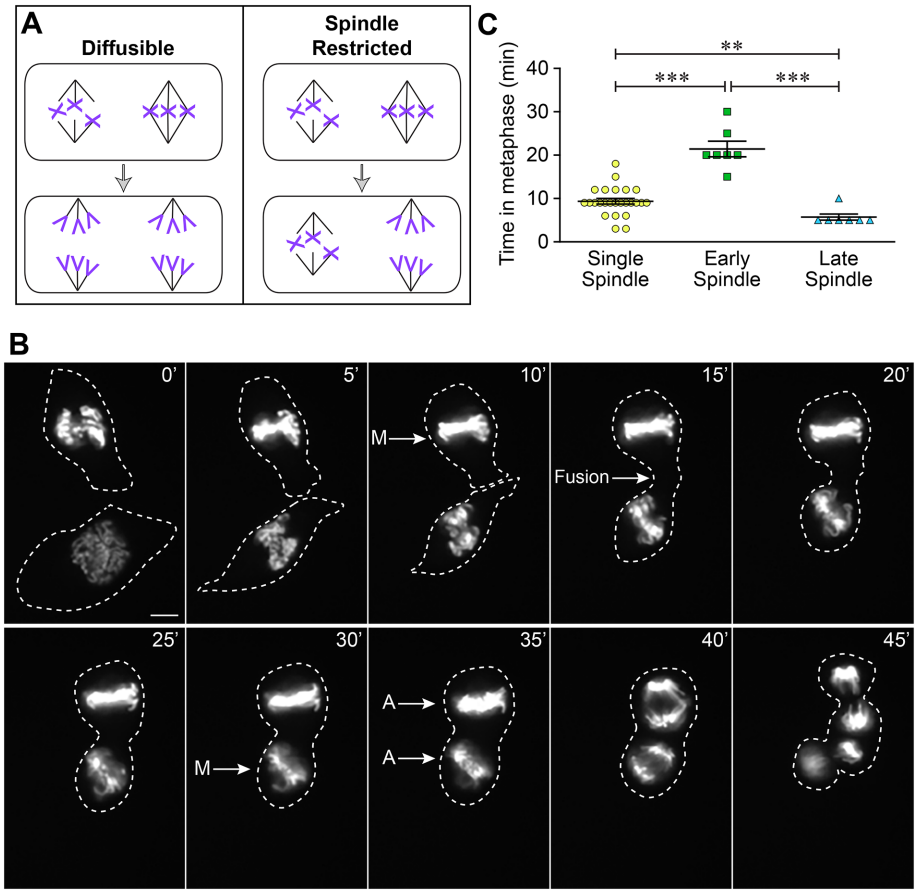

FIGURE 2: Spindles wait to initiate anaphase together. (A) Models describing our predictions that diffusible "wait anaphase" signals should synchronize anaphase onset of multiple mitotic spindle compartments (left), but spindle-restricted signals should not (right). (B) Representative time-course of two mitotic cells stably expressing H2B-GFP fusing (at 15 min) and undergoing synchronous anaphase (A, anaphase spindle; M, metaphase spindle). (C) Quantification of metaphase duration in early and late spindles in cells undergoing synchronous anaphase (see the text). Data set of RO3306 synchronized, fusion medium–exposed cells from Figure 1B is included for comparative purposes (Single Spindle). Statistical significance was determined using unpaired *t* tests (**$p \leq 0.001$; ***$p \leq 0.0001$).

barriers between two spindle compartments within a shared cytoplasm (Figure 3A, ii and iii, bottom). However, if these signals are indeed diffusible in the cytoplasm, then both interspindle distance and degree of cytoplasmic constriction would be expected to alter the extent over which the inhibitory complexes can act (Figure 3A, ii and iii, top). We measured the interspindle distance in fused cells and correlated this distance to the anaphase behavior in each cell (synchronous vs. asynchronous). We found that synchronous anaphase spindles were typically in close proximity to one other within the fused cell. Specifically, we found that spindle compartments <~30 µm apart underwent synchronous anaphase, whereas spindles >~40 µm apart underwent asynchronous anaphase (Figure 3C). Of interest, we found that asynchronous cells possessed narrower cytoplasmic constrictions than synchronous cells (Figure 3, B and E). We quantified the extent of this constriction for each cell by calculating their constriction coefficient, defined as the ratio between the narrowest and widest points for a given fused cell (Figure 3A; c, constriction; w, widest point). Our live-cell studies revealed that these constrictions develop as mitosis progresses (Figure 3D). In the first frame of Figure 3D (time = 0 min), there is a modest degree of constriction (constriction coefficient at $t_{0'} = 0.36$), and both nuclei are in prophase. As mitosis progresses, the constriction becomes more pronounced (constriction coefficient at $t_{50'} = 0.15$), and the spindle compartments undergo asynchronous anaphase (left spindle at 50 min; right spindle at 70 min; see arrow at A). We interpret the asynchronous anaphase onset in such cells to be a consequence of the presence of a barrier to diffusion of "wait anaphase" signals between the two spindles.

As further evidence that "wait anaphase" signals are subject to diffusion barriers, we observed both synchrony and asynchrony of anaphase in a single fused trinucleate cell (Figure 4). Two of the nuclei remained within 13 µm of one another, whereas the third nucleus was situated ~68 µm away from the other two. All three nuclei entered mitosis at the same time, as evidenced by the synchrony of NEBD (at 20 min). This was expected because it is known that cyclin B/CDK1 is a powerful inducer of mitosis, even in large syncytia containing multiple nuclei (Johnson and Rao, 1970; Johnson *et al.*, 1970; Rao and Johnson, 1970). After NEBD, all three nuclei formed distinct mitotic spindles. As was commonly seen throughout these experiments, a cellular constriction formed between the two closely apposed spindles and the third distant spindle. At 65 min after NEBD (at 85 min), the closely apposed spindles underwent synchronous anaphase. However, the distant spindle, now separated by a cytoplasmic constriction, did not undergo anaphase until 140 min, ~120 min after NEBD, and 55 min after the other spindles. These observations are consistent with our observations in bispindled cells and lend additional support to the notion that "wait anaphase" signals are indeed diffusible. Thus, factors that can influence the extent of SAC protein diffusion, including interspindle distance, a diffusion barrier such as a cytoplasmic constriction, or a combination of both can provide spindles with autonomy with respect to mitotic progression.

Dilution of "wait anaphase" complexes affects the strength of SAC signaling

Whereas spindle compartment–restricted "wait anaphase" complexes should be unaffected by changes in cytoplasmic volume, diffusible "wait anaphase" signals should be sensitive to cytoplasmic dilution. We predicted that if cytoplasmic "wait anaphase" complexes were diluted by additional nonmitotic cytoplasm, the SAC might become compromised, causing spindle compartments to prematurely exit mitosis (Howell *et al.*, 2000; Galli and Morgan, 2016; Figure 5A). To test this prediction, we exposed an asynchronous population of PtK1 cells to fusion media in order to observe fusion events between mitotic and interphase cells. Indeed, the majority of these fusion events resulted in premature mitotic exit (Figure 5, B and C; and unpublished data). We observed two types of aberrant mitotic exit resulting from fusion of a mitotic cell with a nonmitotic cell. The first and most common type of premature mitotic exit

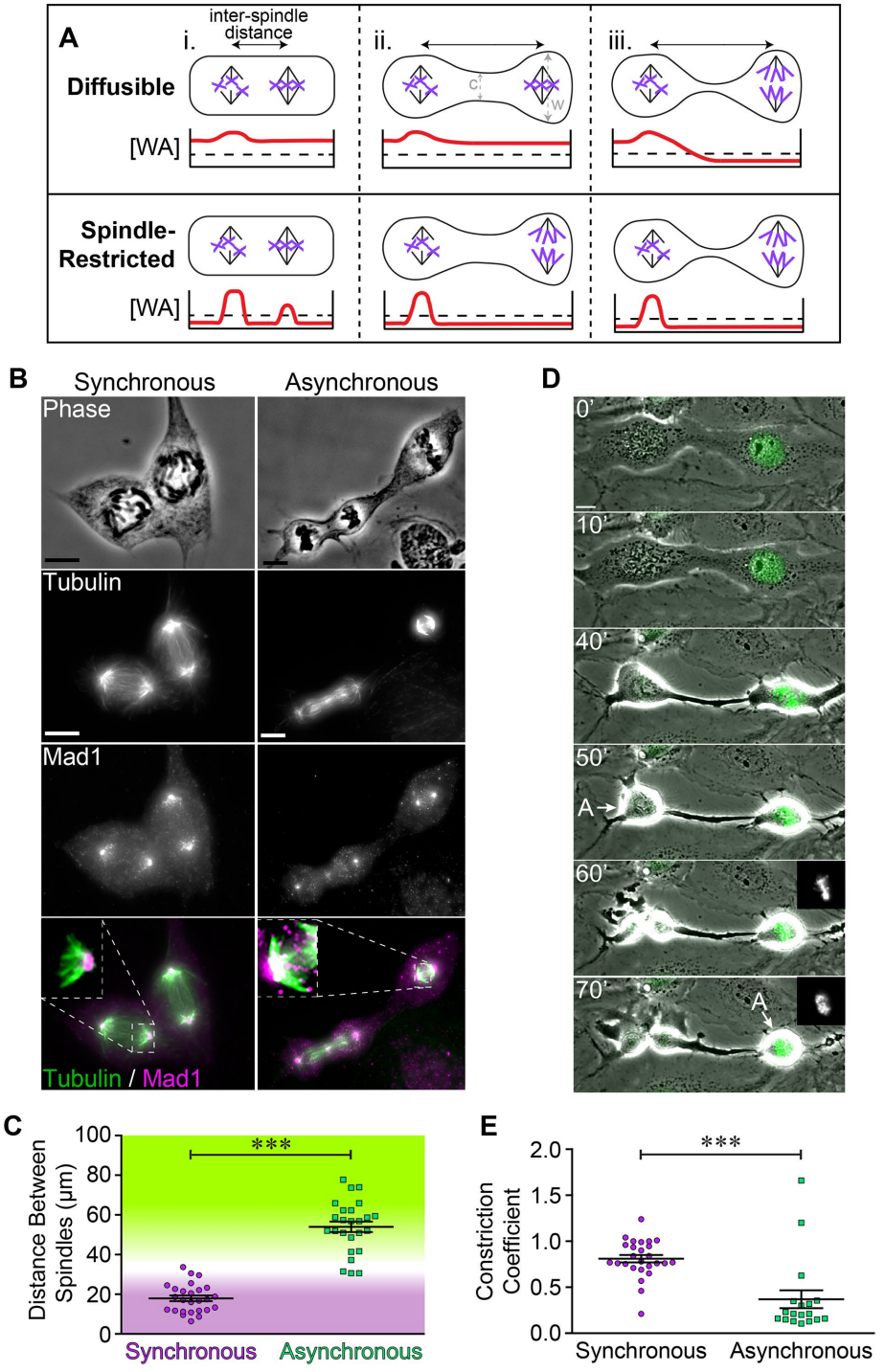

FIGURE 3: Synchrony of anaphase onset correlates with reduced interspindle distance and cytoplasmic constriction in bi-spindled cells. (A) Model describing our predictions for how diffusible and spindle-restricted wait anaphase complexes would respond to three different cellular scenarios: (i) one in which two spindles are in relative close proximity and have no cellular constriction; (ii) one in which the spindles are relatively distal and have only a moderate cellular constriction; and (iii) one in which the spindles are relatively distal and have a severe cellular constriction. Cartoon graphs represent the concentration of "wait anaphase" complexes [WA] across the length of a bi-spindled cell (red line). The black dashed line denotes the threshold of "wait anaphase" signals needed to prevent the spindle compartments from initiating anaphase. For diffusible complexes, less constriction (i and ii) will allow the levels of active MCC complexes to remain above the threshold necessary to prevent mitotic exit across the entirety of the cell. However, when the cell is constricted to the point of restricting diffusion from one side of the cell to the other (iii), active MCC complexes are restricted in the extent of their reach and thus cannot influence the behavior of multiple spindle compartments. In iii, top, a spindle compartment in metaphase (right) was not prevented from entering anaphase because the constriction impeded the diffusion of active MCC molecules from the spindle compartment in prometaphase (left). If MCC molecules are restricted to the spindle compartment, spindle behavior would be autonomous to each spindle regardless of any barriers to diffusion between the two compartments (c, constriction, or narrowest point for a given cell; w, widest dimension for a given cell). (B) Micrographs of fused, fixed, and stained (as indicated) mitotic cells with two spindles undergoing either synchronous anaphase (left) or asynchronous anaphase (right). (C) Anaphase behavior in relation to interspindle distance, as measured from fixed cells. Purple and green gradients indicate approximate distance thresholds in which spindles have a high probability of being synchronous (<~30 μm) or asynchronous (>~40 μm), respectively. (D) Representative time-lapse images of a fused cell (with one nucleus stably expressing H2B-GFP) developing a cytoplasmic constriction. Insets at 60 and 70 min depict H2B-GFP fluorescence. (E) Anaphase behavior in relation to the constriction coefficient, as measured from fixed cells. Statistical significance was determined using unpaired *t* tests. Scale bars, 10 μm. ***$p \leq 0.0001$.

consisted of sister chromatid separation and chromosome decondensation without chromosome alignment (Figure 5B). Figure 5B shows an example of this type of mitotic exit occurring in a fused cell containing three nuclei, each in different phases of the cell cycle: the top spindle compartment is in prometaphase (PM), the bottom left spindle compartment is in late anaphase (A), and the bottom right nucleus is in interphase (I). Soon after the fusion event, the prometaphase spindle compartment can be observed exiting mitosis without aligning its chromosomes or initiating anaphase (see "Exit" at 30 min). The other, less commonly observed example of premature mitotic exit consisted of anaphase onset in the presence of unaligned chromosomes (Figure 5C). In this example, which shows a fused cell containing a prometaphase nucleus (PM, top) and two interphase nuclei (I, bottom), the prophase nucleus forms a bipolar spindle ("BPS" at 65 min; unlike the example shown in Figure 5B). However, anaphase onset (A at 70 min) occurs in spite of the presence of unaligned chromosomes (arrowheads at 70 min). Of interest but not surprisingly, the two interphase nuclei undergo premature chromosome condensation ("PCC" at 65 min; a sign of mitotic entry), similar to our observations described earlier (see Figure 4

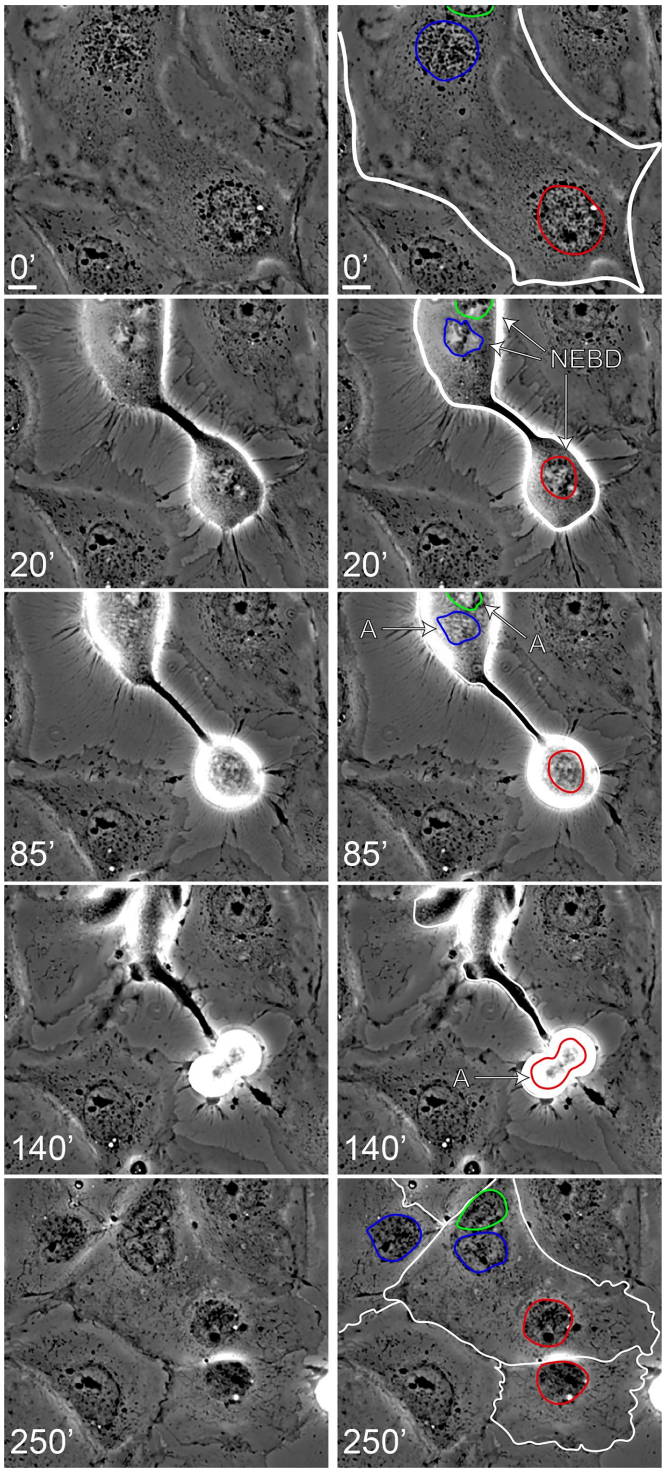

FIGURE 4: Anaphase synchrony is distance dependent. In this example of a trinucleate cell (unannotated on the left, annotated on the right), all nuclei are in prophase at 0 min. Two nuclei are clustered at the top of the cell (bounded by blue and green outlines in annotated images on the right). The green nucleus is just out of the field of view at 0 min but moves into view in subsequent frames. The third nucleus is at the bottom of the cell (bounded by a red outline). By 20 min, all three nuclei have initiated NEBD, a cellular constriction has formed between the closely apposed nuclei (blue and green) and the distal nucleus (red), and the blue and green nuclei begin to align their chromosomes. At 85 min, the blue and green spindle compartments initiate anaphase synchronously, while the red spindle compartment remains in prometaphase. At 140 min, the red spindle compartment initiates anaphase, 55 min after the blue and green spindle compartments initiated anaphase. At 250 min, all cell products from the three mitotic spindles reenter interphase. The nuclei produced from each mitotic spindle at 250 min are outlined in their respective colors. One of the green daughter cells is out of frame. Scale bars, 10 μm.

and associated discussion). We interpret the premature mitotic exit observed in these fused cells to be indicative of perturbed SAC activity and attribute this dysfunction to the dilution of cytoplasmic "wait anaphase" signals. Although our data suggest a model in which mitotic exit results from dilution of active "wait anaphase" complexes, we cannot formally rule out the possibility that cytoplasmic factors exist in the interphase cells that could potentially promote the disassembly of MCC complexes and thereby promote premature mitotic exit.

Of note, when we performed the same experiment with RO3306 synchronized cells (i.e., arrested at G2/M), mitotic spindle compartments waited at metaphase for the interphase nuclei to enter mitosis and align their chromosomes before a synchronous anaphase onset (Figure 5D). This is not unexpected because RO3306 leads to the accumulation of cells in late G2, a point at which cells are competent to enter mitosis and generate active MCC complexes (Fraschini *et al.*, 2001; Sudakin *et al.*, 2001; Maciejowski *et al.*, 2010; Rodriguez-Bravo *et al.*, 2014).

DISCUSSION

In this study, we used cell fusion experiments to test the hypothesis that "wait anaphase" signals are restricted to the spindle compartment within which they are generated. This hypothesis, which was originally proposed by Rieder *et al.* (1997), has remained a prominent theory in the field of SAC signaling and mitosis. However, as we have gained a more detailed understanding of SAC signaling and the molecules involved, it is difficult to conceive of a mechanism by which SAC components would be restricted to the spindle region and thus be excluded from the cytoplasm outside of the spindle. We therefore directly tested the hypothesis using predictions of how fused, multispindled cells would behave during mitosis if the "wait anaphase" signals were spindle restricted versus being diffusible. If such signals are indeed spindle-restricted, we predicted that 1) spindle compartments in a shared cytoplasm would progress through mitosis autonomously, 2) spindle compartments in a shared cytoplasm would be unaffected by diffusion barriers, and 3) mitotic progression would be unaffected by the introduction of additional cytoplasmic volume. For each of these predictions, we observed cellular behaviors that instead indicate that "wait anaphase" signals are in fact diffusible outside the confines of the spindle compartment. Specifically, we found that 1) spindle compartments within a shared cytoplasm exhibit synchronized mitotic progression (i.e., undergo simultaneous anaphase onset), 2) anaphase synchrony is disrupted in the presence of a diffusion barrier such as a cytoplasmic constriction, and 3) spindle compartments prematurely exit mitosis when exposed to nonmitotic cytoplasm via cell fusion with an interphase cell. These findings provide new insight into the molecular biology of the SAC, with specific attention to where in the cell "wait anaphase" signals are active.

These results have important implications for our understanding of mitotic checkpoint dysfunction during embryonic cell division. The embryonic cells of most animal species lack a robust SAC until embryos reach the midblastula transition (Hara *et al.*, 1980;

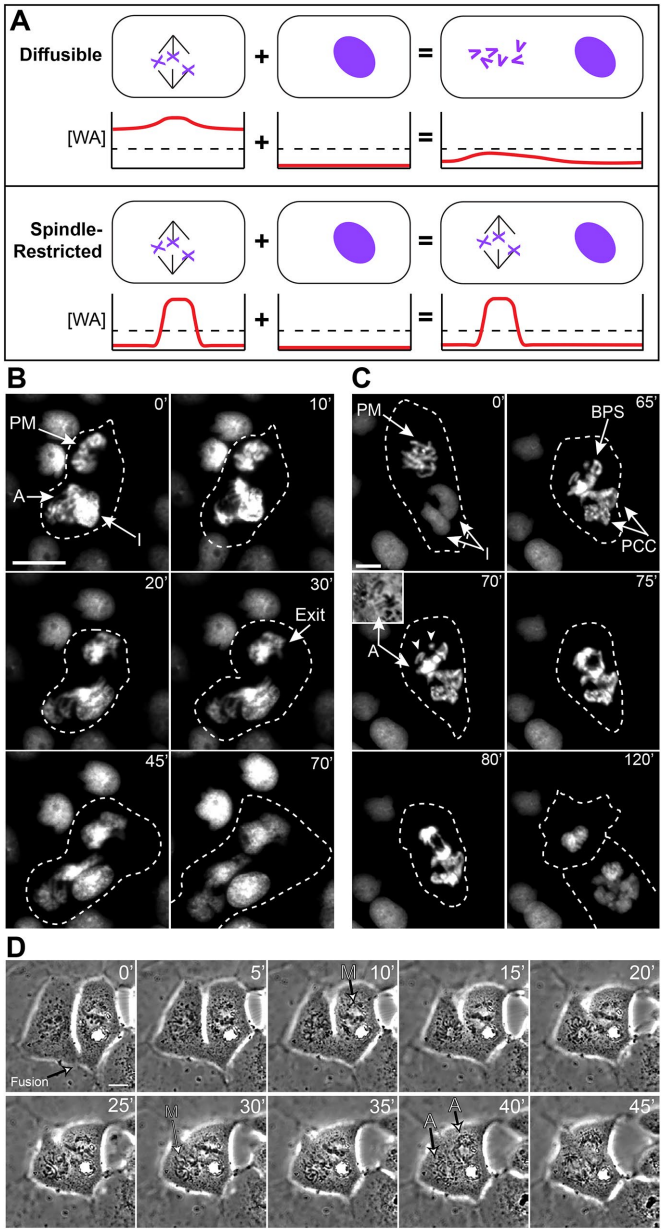

FIGURE 5: Dilution of "wait anaphase" signals affects SAC activity. (A) Model describing how diffusible and spindle-restricted wait anaphase complexes should respond to cytoplasmic dilution. As in Figure 3A, cartoon graphs depict the concentration of "wait anaphase" complexes across the cell (red line). The threshold of "wait anaphase" signal concentration required to prevent anaphase onset is denoted with a black dashed line. If "wait anaphase" signals are diffusible, the addition of nonmitotic cytoplasm via fusion with a nonmitotic cell (cells on the right in each example) would dilute the overall concentration of "wait anaphase" complexes. This would result in premature mitotic exit (i.e., spindle disassembly, as depicted in the fused cell cartoon, top) because the concentration of "wait anaphase" signaling molecules (i.e., active MCC complexes) drops below the threshold required for SAC-mediated arrest. Conversely, if active MCC complexes are restricted to the spindle compartment, addition of cytoplasm would not perturb SAC activity, and each spindle compartment would progress through mitosis with normal timing. (B, C) Representative time-lapse images of fused H2B-GFP–expressing cells in which mitotic spindle compartments prematurely exit mitosis after fusion with nonmitotic cells (see the text). Inset in C (at 70 min) shows separation of sister chromatids as an indicator of anaphase onset. (D) Representative time-lapse images of fused RO3306 synchronized cells, demonstrating that cells already in mitosis and G2 before cell fusion undergo synchronous anaphase. In this binucleate cell, the spindle compartment in metaphase (M, at 10 min) delays anaphase onset until the interphase nucleus (left; presumably in G2 phase at point of fusion) enters mitosis (30 min) and aligns its chromosomes, at which point both spindle compartments initiate anaphase (40 min). Scale bars, 10 μm. A, anaphase; BPS, bipolar spindle; Exit, mitotic exit; I, interphase; M, metaphase; PCC, premature chromosome condensation; PM, prometaphase; arrowheads, unaligned chromosomes.

Zhang *et al.*, 2015; Galli and Morgan, 2016). As a result, chromosome segregation errors occur at a higher frequency early in embryogenesis due to premature mitotic exit (Daphnis *et al.*, 2005). A predominant hypothesis to explain deficient checkpoint function in these cells is that the large cytoplasmic volume of embryonic cells might dilute the inhibitory "wait anaphase" signal, thus reducing the strength of the SAC. Thus, a defined kinetochore:cytoplasm ratio is required to ensure appropriately timed and error-free mitoses. This model has gained support from a recent study that measured checkpoint strength in the embryos of the nematode *Caenorhabditis elegans* (Galli and Morgan, 2016). As is the case in many animals, the early embryonic cells of *C. elegans* divide such that the cellular volume is reduced at each round of division (i.e., they undergo reductive divisions). The resulting cells are of equal ploidy (and thus contain equal numbers of kinetochores) but are smaller in volume. As cell volume decreases, the ability of cells to maintain a nocodazole-induced mitotic arrest improves (Galli and Morgan, 2016). A critical assumption of this kinetochore:cytoplasm hypothesis is that inhibitory "wait anaphase" signals can diffuse outside of the confines of the spindle and into the cytoplasm. It is unclear how this signal could become diluted if it was restricted to the spindle compartment. Our studies offer clarification as to how dilution of "wait anaphase" signals could occur.

Within the human body, there are very few cell types that maintain a multinucleate state (e.g., skeletal and placental tissue). However, our findings that "wait anaphase" signals can be communicated through the cytoplasm between two spindle compartments have direct application to the behavior of the SAC in mononucleate cells. For example, during mitosis, it is not uncommon for chromosomes to become isolated outside the confines of the spindle compartment. These chromosomes, whether they are stuck behind the spindle poles or simply distal from the spindle compartment, are known to be competent to 1) recruit SAC components such as Mad1 and 2) cause a delay in mitotic progression (Collin *et al.*, 2013). Our results here reveal the physical basis for this delay, which we propose is due to the diffusion of active MCC complexes away from these unattached chromosomes throughout the cell, which consequently globally inhibit the APC/C.

MATERIALS AND METHODS

Cell culture, synchronization, and stable cell line generation

PtK1 cells were cultured in 5% CO_2 in Ham's F-12 medium supplemented with 15% fetal bovine serum (FBS) and 1% penicillin/streptomycin. PtK1 cells were partially synchronized using the CDK1 inhibitor RO3306 (Sigma-Aldrich). Cells were treated with 9 μM RO3306 (in dimethyl sulfoxide [DMSO]) for 20 h and subsequently released by washing with phosphate-buffered saline (PBS) four or five times and then replaced into drug-free medium. We used the

following method to generate stable H2B-green fluorescent protein (GFP)–expressing PtK1 cells. HEK-293T (the viral host) cells were transfected with pPax2 and pMD2.G viral packaging plasmids along with pGK:H2B-eGFP vector (Addgene). PtK1 cells were then infected with resulting virus and cultured for 24 h. Subsequently, fresh virus was added to these cells, which were then cultured for an additional 24 h. At 48 h after the initial infection, cells were grown in selection media (Ham's F-12 supplemented with hygromycin B). Single colonies were then picked, expanded in 24-well plates in selection medium, and then visually screened via fluorescence microscopy for H2B-GFP expression.

Cell fusion

PtK1 cells were fused as previously described (Peterson and Berns, 1979). Fusion medium was made by melting 8 g of polyethylene glycol 3500 (PEG) in an autoclave, cooling the molten PEG to 45°C, and diluting with 2 ml of DMSO and 10 ml of prewarmed (37°C) DMEM without FBS. To fuse cells, normal growth medium was aspirated, and fusion medium was added to the cells for 35–45 s. Cells were then repeatedly washed with PBS (four or five times) until residual fusion medium was removed and replaced into Ham's F-12 medium supplemented with 15% FBS and 1% penicillin/streptomycin.

Immunofluorescence

Coverslip cultures of fused PtK1 cells were rinsed in PHEM buffer (60 mM 1,4-piperazinediethanesulfonic acid, 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 10 mM ethylene glycol tetraacetic acid, and 8 mM $MgSO_4$, pH 7.0), lysed in PHEM buffer supplemented with 1% Triton X-100 for 1 min, and then fixed in 4% paraformaldehyde for 20 min. Cells were rinsed 3 × 5 min in PHEM buffer supplemented with 0.5% Triton X-100 and then blocked for 1 h at room temperature with 10% boiled donkey serum (BDS) in PHEM. Primary antibodies were diluted in 5% BDS in PHEM as follows: mouse anti–α-tubulin, 1:300 (T6199; Sigma-Aldrich) and rabbit anti-Mad1 (1:500; 109519, lot 40030; Gene Tex). Cells were incubated with primary antibodies overnight at 4°C and then rinsed 3 × 5 min in PHEM supplemented with 0.05% Triton X-100. Cells were incubated with appropriate secondary antibodies (donkey anti-mouse or donkey anti-rabbit) conjugated to Alexa Fluor 647 or 555 (Jackson ImmunoResearch Laboratories) diluted 1:300 in 5% BDS in PHEM for 45 min at room temperature. Cells were then rinsed with PHEM supplemented with 0.05% Triton X-100 4 × 5 min, rinsed once with PHEM, and mounted onto slides using mounting medium (20 mM Tris, pH 8.0, 0.5% *N*-propyl gallate, and 90% glycerol). Coverslips were sealed to the slides using fingernail polish.

Microscopy and analysis

Cells were imaged with a DeltaVision Personal DV (Applied Precision) imaging system equipped with a 40×/0.75 numerical aperture Ph UPlanFL objective (Olympus) and a CoolSNAP HQ2 (Photometrics/Roper Scientific) camera. The system was controlled with softWoRx acquisition software (Applied Precision). Images for fixed-cell experiments (Figure 3, B, C, and E) were acquired as *z*-stacks with 200-nm step sizes. For live-cell experiments, (Figures 1, 2, 3D, 4, and 5), cells were maintained at 37°C using an environmental chamber (Precision Control, Seattle, WA), and a single *z*-plane was acquired every 3 or 5 min, depending on the experiment.

Measuring interspindle distance and constriction coefficients

Interspindle distance was measured in Fiji (ImageJ; National Institutes of Health, Bethesda, MD) by determining the distance (i.e., the most direct intracellular path) between the centroid of each spindle (centroids were approximated by determining the midpoint between spindle poles). Because PtK1 cells remain flat during mitosis, distances in the *z*-dimension are negligible and were not used (unpublished data). We noted that fused asynchronous cells frequently displayed a severe constriction between the two spindles. When the constriction was very narrow (i.e., constriction coefficient ≤0.1; see Figure 3Aii), it became difficult to determine whether cells were fused. Thus we included only cells in which the constriction width was ≥10% of the maximum width of the cells (i.e., constriction coefficient >0.1). Constriction coefficient values were calculated by dividing the width of the narrowest point of the constriction (c) by the width of the widest point (w) in the cells (Figure 3Aii).

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