Polar Ejection Forces Promote the Conversion from Lateral to End-on Kinetochore-Microtubule Attachments on Mono-oriented Chromosomes

Graphical Abstract

Highlights

- Spindle assembly checkpoint (SAC) can be satisfied after a delay in cells with mono-oriented chromosomes
- Mono-oriented chromosomes experience intra-kinetochore stretch
- Polar ejection forces promote SAC satisfaction independently of bi-orientation
- Polar ejection forces promote the conversion from lateral to end-on attachments

In Brief

Tension on bi-oriented chromosomes plays a role in the stabilization of kinetochore-microtubule attachments. However, how kinetochore-microtubule attachments on mono-oriented chromosomes are first stabilized in the absence of tension remained unknown. Drpic et al. now show that polar ejection forces promote the transition from lateral to end-on attachments on mono-oriented chromosomes.

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Polar Ejection Forces Promote the Conversion from Lateral to End-on Kinetochore-Microtubule Attachments on Mono-oriented Chromosomes

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SUMMARY

Chromosome bi-orientation occurs after conversion of initial lateral attachments between kinetochores and spindle microtubules into stable end-on attachments near the cell equator. After bi-orientation, chromosomes experience tension from spindle forces, which plays a key role in the stabilization of correct kinetochore-microtubule attachments. However, how end-on kinetochore-microtubule attachments are first stabilized in the absence of tension remains a key unanswered question. To address this, we generated Drosophila S2 cells undergoing mitosis with unreplicated genomes (SMUGs). SMUGs retained single condensed chromatids that attached laterally to spindle microtubules. Over time, laterally attached kinetochores converted into end-on attachments and experienced intra-kinetochore stretch/structural deformation, and SMUGs eventually exited a delayed mitosis with mono-oriented chromosomes after satisfying the spindle-assembly checkpoint (SAC). Polar ejection forces (PEFs) generated by Chromokinesins promoted the conversion from lateral to end-on kinetochore-microtubule attachments that satisfied the SAC in SMUGs. Thus, PEFs convert lateral to stable end-on kinetochore-microtubule attachments, independently of chromosome bi-orientation.

INTRODUCTION

During spindle assembly, the initial lateral interactions between chromosomes and microtubules are converted into stable end-on kinetochore-microtubule attachments that lead to chromosome bi-orientation (Magidson et al., 2011). After chromosome bi-orientation, the opposing spindle forces generate tension on centromeres that is important for the stabilization of correct kinetochore–microtubule attachments required for error-free chromosome segregation (Nicklas and Koch, 1969; Nicklas and Ward, 1994). Tension has also been shown to be sufficient to satisfy the spindle-assembly checkpoint (SAC) (Li and Nicklas, 1995), a surveillance mechanism that ensures that all chromosomes are attached to spindle microtubules before anaphase onset (Foley and Kapoor, 2013). Tension from spindle forces affects kinetochore chemistry through changes in phosphorylation of “tension-sensitive” proteins at kinetochores (Gorbosky and Ricketts, 1993; Nicklas et al., 1995). Aurora B, a mitotic kinase present on centromeres, plays a critical role in tension sensing and error correction (Biggins and Murray, 2001; Cheeseman et al., 2002; Lampson et al., 2004) by phosphorylating key substrates at the kinetochore-microtubule interface, such as the KMN network, in response to tension on bi-oriented chromosomes (DeLuca et al., 2006; Liu et al., 2009; Wang et al., 2011; Welburn et al., 2010). Importantly, recent works in human and Drosophila cells have shown that even in the absence of centromeric tension, an intra-kinetochore stretch or structural deformation is sufficient to satisfy the SAC (Maresca and Salmon, 2009; Uchida et al., 2009). However, the underlying mechanism remains unclear.

Chromokinesins are microtubule plus-end-directed motor proteins present on the chromosome arms harboring both chromatin- and microtubule-binding domains. As a consequence of their motor activities, chromokinesins move chromosomes away from the poles by generating random polar ejection forces (PEFs) (Barisic et al., 2014; Brouhard and Hunt, 2005; Levesque and Compton, 2001; Rieder et al., 1986; Wandke et al., 2012; Yajima et al., 2003). Recently, elevated PEFs were shown to stabilize erroneous kinetochore-microtubule attachments (Cane et al., 2013), suggesting a role in the stabilization of kinetochore-microtubule attachments. Here, we found that Chromokinesin-mediated PEFs promote the conversion from lateral to
stable end-on kinetochore-microtubule attachments on monoriented chromosomes. These findings contribute to explain how initial end-on kinetochore-microtubule attachments are stabilized before bi-orientation.

RESULTS

The SAC Is Satisfied in Cells with Single Chromatids after a Mitotic Delay

To investigate which factors are responsible for kinetochore-microtubule attachment stability before bi-orientation, we established a system in Drosophila S2 cells undergoing mitosis with unreplicated genomes (SMUGs) (Drpic et al., 2013). This was achieved by RNAi-mediated depletion of Double parked (Dup), a conserved protein required for the initiation of DNA replication and post-replication checkpoint response (Whittaker et al., 2000). The main advantage of this system when compared to mammalian cells undergoing MUGs (Brinkley et al., 1988; O’Connell et al., 2009) is that SMUGs preserve their unreplicated genetic material condensed into single chromatids, which never experience bi-orientation due to the absence of sister kinetochores (Drpic et al., 2013). Thus, the function of individual kinetochores in SMUGs can be investigated in their native chromatic context.

Spinning-disk confocal live-cell imaging revealed that single chromatids in SMUGs were scattered along the spindle. Because of their low chromosome number, the status of kinetochore-microtubule attachments could be inferred by careful inspection of the respective z-sections (see Experimental Procedures). This indicated that SMUGs established mainly lateral and only few merotelic kinetochore-microtubule attachments. For instance, 20 min after nuclear envelope breakdown (NEB) we found that, on average, 8.0 ± 1.6 kinetochores per cell were laterally attached and 3.0 ± 0.82 kinetochores established merotelic attachments (mean ± SD, n = 5 cells; Figures 1A and S1A; Movie S1). Consequently, SMUGs significantly delayed mitotic exit (t = 111 ± 43 min, mean ± SD, n = 11 cells, p < 0.001, t test) when compared to control cells (t = 31 ± 8 min, mean ± SD, n = 11 cells; Figures 1A and 1C; Movie S1). Indeed, while cyclin B1 levels abruptly decreased at the metaphase-anaphase transition in control cells, cyclin B1 levels decreased more slowly over time in SMUGs (Figures S1E and S1F), suggesting a delay in SAC satisfaction (see also Mirkovic et al., 2015 in this issue of Cell Reports). To investigate whether the delayed mitotic exit in SMUGs is SAC dependent, we co-depleted Mad2 and Dup by RNAi (Figures 1C, S1B, and S1C). We found that, similar to control cells, Mad2 co-depletion overcomes the mitotic delay in SMUGs (Mad2/Dup-depleted cells: t = 22.1 ± 6.0 min, mean ± SD, n = 31 cells; Mad2-depleted cells: t = 18.0 ± 5.6 min, mean ± SD, n = 19 cells), indicating that the mitotic delay in SMUGs is SAC dependent.

Next, we tested SAC response in SMUGs by adding colchicine immediately after NEB to generate unattached kinetochores and monitored mitotic progression by live-cell imaging. Both control cells and SMUGs were arrested in mitosis for more than 10 hr before undergoing slippage (Rieder and Maiato, 2004) (control t = 18.4 ± 1.23 hr, mean ± SD, n = 7 cells; SMUGs t = 10.4 ± 2.6 hr, mean ± SD, n = 24 cells; Figures 1B and 1C). These results indicate that SMUGs have an active SAC, which is, however, less robust than in control cells. Interestingly, the total levels of Mad2 and the recruitment of Mad2 and active Aurora B to unattached kinetochores in SMUGs were unaltered relative to controls; Figures S1D and S2A–S2D). Thus, despite normal SAC signaling at individual kinetochores, the number of cumulative unattached kinetochores that are able to inhibit the Anaphase Promoting Complex/Cyclosome (APC/C) in SMUGs is reduced by half relative to controls cells. This explains the weakened SAC response in SMUGs and is in line with previous reports in human cells (Collin et al., 2013; Dick and Gerlich, 2013). Importantly, these data strongly suggest that SMUGs normally exit mitosis after SAC satisfaction, as they took more than five times longer to slip out of mitosis in the presence of colchicine.

To directly test whether SMUGs satisfy the SAC after a mitotic delay, we investigated the behavior of another SAC protein, BubR1, using live-cell imaging of SMUGs stably expressing BubR1-mCherry/α-tubulin-GFP. BubR1 is normally recruited to unattached kinetochores and its levels decrease significantly as chromosomes bi-orient, becoming undetectable on anaphase kinetochores (Howell et al., 2004; Maiato et al., 2002). In contrast, BubR1 remains associated with kinetochores in cells that slip out of mitosis without satisfying the SAC (Brito and Rieder, 2006). We found that, despite of a mitotic delay, SMUGs lost BubR1 from kinetochores just before exiting from mitosis (Figures 1D–1F and Movie S2). This demonstrates that the SAC in SMUGs with single chromatids can be satisfied without bi-orientation.

Single Chromatids in SMUGs Experience Intra-kinetochore Stretch/Structural Deformation after a Mitotic Delay

Intra-kinetochore stretch or structural deformation is sufficient to satisfy the SAC even with reduced centromeric tension (Maresca and Salmon, 2009; Uchida et al., 2009). To investigate whether

Figure 1. Cells with Single Chromatids Satisfy the SAC after a Mitotic Delay

(A) Live-cell imaging of Drosophila S2 cells (control and Dup-depleted) stably expressing H2B-GFP and mCherry-α-tubulin. Dashed box indicates a single, condensed chromosome.

(B) Similar conditions, but in which cells were treated with 200 μM colchicine immediately after NEB.

(C) Quantification of mitotic duration (control n = 11 cells; Dup-depleted n = 11 cells; control cells treated with colchicine n = 7 cells; Dup-depleted cells treated with colchicine n = 24 cells; Mad2-depleted cells treated with colchicine n = 19 cells; Mad2/Dup-depleted cells treated with colchicine, n = 31 cells).

(D) Live-cell imaging of S2 cells stably expressing BubR1-mCherry and GFP-α-tubulin.

(E and F) Quantification of the number of BubR1 positive kinetochores during normal mitosis (n = 10 cells) and SMUGs (n = 10 cells). Zero time point refers to anaphase onset.

IIIp < 0.001. Black lines indicate individual cells and red lines represent the average. Error bars, SD. Time = hr:min. Scale bar, 5 μm. See also Figures S1 and S2 and Movie S1.
SMUGs experience intra-kinetochore stretch/structural deformation, we measured the absolute distance between the inner kinetochore protein Cid-mCherry and the outer kinetochore protein Ndc80-GFP (Maresca and Salmon, 2009) at individual kinetochores (see Experimental Procedures) from control cells treated with colchicine (reference for relaxed kinetochores) or MG132 (reference for bi-oriented chromosomes under tension), as well as from Dup-depleted cells treated with MG132 for 2 hr (to normalize the mitotic delay). We found that under these conditions single chromatids in SMUGs experienced a significant intra-kinetochore stretch/structural deformation relative to relaxed kinetochores (Mann-Whitney rank-sum test, p < 0.001) that was almost comparable to bi-oriented chromosomes under tension (Figures 2A and 2C). In line with these measurements, we further observed intermediate levels of Aurora B-mediated phosphorylation of the outer kinetochore protein KNL1 (Welburn et al., 2010) relative to unattached controls and bi-oriented chromosomes (Figures 2B and 2C), suggesting that intra-kinetochore stretch/structural deformation positively correlates with kinetochore-microtubule attachment stability. Taken together, these data indicate that single chromatids in SMUGs experience sufficient intra-kinetochore stretch/structural deformation to satisfy the SAC.

PEFs Stabilize Kinetochore-Microtubule Attachments and Promote SAC Satisfaction Independently of Chromosome Bi-orientation

Elevated PEFs on chromosome arms after overexpression of the Chromokinesin Nod lead to the stabilization of syntelic kinetochore-microtubule attachments in Drosophila S2 cells (Cane et al., 2013). To test whether the kinetochore-microtubule stabilizing role of PEFs is involved in SAC satisfaction in SMUGs, we co-depleted Dup and Nod. This resulted in a SAC-dependent increase in mitotic duration when compared to Dup-depleted cells (t = 208 ± 109 min, mean ± SD, n = 25 cells, p = 0.007, t test; Figures 3B and 3D; Movie S3). Co-depletion of both Chromokinesins, Nod and Klp3A, with Dup caused an even longer mitotic delay (t = 304 ± 66 min, mean ± SD, n = 8 cells, p ≤ 0.001, t test; Figures 3D and S3E). Interestingly, Nod depletion in control cells caused chromosome alignment defects and also significantly increased the duration of mitosis (t = 44 ± 12 min, mean ± SD, n = 26, p = 0.005, Mann-Whitney rank-sum test; Figures 3A and 3D; Movie S3), in line with previous findings in human cells (Levesque and Compton, 2001; Magidson et al., 2011). This phenotype was exacerbated when Nod and Klp3A were co-depleted (t = 62 ± 29 min, mean ± SD, n = 20, p = 0.003, t test; Figures 3D and S3E), suggesting that PEFs play an important role in the stabilization of kinetochore-microtubule attachments during a normal mitosis. Thus, in the absence of Chromokinesin-mediated PEFs, SAC satisfaction is delayed and the delay is more pronounced in the absence of chromosome bi-orientation.

One prediction from these data is that elevated PEFs should promote the stabilization of kinetochore-microtubule attachments and consequently accelerate SAC satisfaction in SMUGs. To test this, we overexpressed Nod-mCherry in Dup-depleted cells stably expressing GFP-α-tubulin (Cane et al., 2013). In agreement with our prediction, Nod overexpression significantly shortened the mitotic duration in Dup-depleted cells (t = 46.5 ± 22 min, mean ± SD, n = 12 cells, p ≤ 0.001, t test; Figures 3C and 3D; Movie S4). In contrast, elevated PEFs caused by Nod overexpression in control cells increased mitotic duration (t = 67 ± 27 min, mean ± SD, n = 22 cells p = 0.003, Mann-Whitney rank-sum test; Figures 3C and 3D; Movie S4), which might be due to random ejection of chromosomes after stabilization of monotelic attachments, thereby preventing bi-orientation and timely SAC satisfaction (Barisic et al., 2014). Overall, these data suggest that Chromokinesin-mediated PEFs promote SAC satisfaction in SMUGs by stabilizing kinetochore-microtubule attachments independently of chromosome bi-orientation.

PEFs Promote the Conversion from Lateral to End-on Kinetochore-Microtubule Attachments on Mono-oriented Chromosomes

HeLa cells undergoing MUGs satisfy the SAC independently of bi-orientation mainly by establishing merotelic attachments (O’Connell et al., 2008). Due to opposite spindle forces, merotelic attachments might cause kinetochore deformation that generates sufficient intra-kinetochore stretch that would satisfy the SAC (Maresca and Salmon, 2009; Uchida et al., 2009). Importantly, the contribution of PEFs for SAC satisfaction could not be investigated in this system because kinetochores detach from chromatin, which remains decondensed during MUGs (Brinkley et al., 1988; O’Connell et al., 2009). Although we cannot exclude that, in addition to PEFs, some merotelic attachments contribute to SAC silencing in SMUGs, these attachments were rare, as indicated by our live-cell recordings and careful inspection of the respective z stacks (Figures 1A and S1A; Movie S1) (see also Mirkovic et al., this issue).

To test whether PEFs are required to satisfy the SAC in SMUGs, independently of chromosome bi-orientation and the establishment of merotelic attachments, we investigated the duration of mitosis in Nod-depleted cells with a monopolar spindle configuration (in which only monotelic attachments can be established), after co-depletion of the Kinesin-5 protein Klp61F by RNAi (Cane et al., 2013) (Figure 4A; Movie S5). We found that SMUGs with monopolar spindles were also able to exit mitosis after a delay (t = 178 ± 59 min, mean ± SD, n = 9; Figure 4A; Movie S5), which was exacerbated after Nod co-depletion (t = 379 min ± 132 min, mean ± SD, n = 4, p = 0.011, Mann-Whitney rank-sum test; Figure 4A; Movie S5). Closer inspection of z stacks from live-cell images of monopolar spindles in SMUGs revealed a clear transition from lateral to end-on kinetochore-microtubule attachments prior to mitotic exit, and the presence of Nod-mediated PEFs promoted this transition (Figures 4B and 4C; Movie S5). Immunofluorescence analysis with a Mad1 antibody confirmed that the percentage of unattached kinetochores in SMUGs with monopolar spindles (35%) increased after Nod depletion (62%, p = 0.028, t test; Figure 4D). Overall, these data demonstrate that Chromokinesin-mediated PEFs promote the conversion from lateral to stable end-on kinetochore-microtubule attachments, independently of bi-orientation and merotely.

DISCUSSION

Chromosome bi-orientation is a critical requirement for accurate chromosome segregation during mitosis and requires that both kinetochores are stably attached to spindle microtubules.
Figure 2. Single Chromatids in SMUGs Experience Intra-kinetochore Stretch after a Mitotic Delay

(A) Fixed control cells stably expressing Cid-mCherry/Ndc80-GFP were treated with colchicine or MG132 (2 hr) and compared with Dup-depleted cells treated with MG132 (2 hr).

(B) Immunofluorescence analysis of Aurora-B phosphorylation of the outer kinetochore protein KNL1 in SMUGs and control cells in the same conditions as in (A).

(C) Quantification of pKNL1 and intra-kinetochore stretch (shift) by measuring absolute distance between red (Cid) and green (Ndc80) centroids in control cells versus SMUGs.
Tension from spindle forces has long been known to stabilize correct kinetochore-microtubule attachments (King and Nicklas, 2000), but how the first end-on attachments are stabilized before the development of tension has remained unknown. Here, we found that PEFs promote the conversion from lateral to stable end-on kinetochore-microtubule attachments on mono-oriented chromosomes. Lateral attachments to spindle microtubules are insensitive to Aurora B activity (Kalantzaki et al., 2015) and are initially mediated by kinetochore Dynein, which is dominant over PEFs at the spindle poles (Barisic et al., 2014) and inhibits the action of the Ndc80 complex required for stable end-on attachments (Cheerambathur et al., 2013). Despite not being dominant at this stage, PEFs promote the exclusion of chromosomes from the central area of the mitotic spindle (Magidson et al., 2011), but chromosomes remain tethered to the microtubule walls by CENP-E/Kinesin-7 (Shrestha and Draviam, 2013), which slides chromosomes preferentially along detyrosinated microtubules toward the spindle equator (Barisic et al., 2015). At the equator PEFs become critical to stabilize end-on kinetochore-microtubule attachments required for chromosome bi-orientation (Barisic et al., 2014; Magidson et al., 2011; Wandke et al., 2012). In this context, our data can be best explained by a model in which the lateral to end-on conversion of kinetochore-microtubule attachments near the equator requires the contribution of Chromokinesin-mediated PEFs acting on the arms of mono-oriented chromosomes to counteract microtubule depolymerization-driven poleward motion. This might generate sufficient intra-kinetochore stretch or structural deformation (Maresca and Salmon, 2009; Uchida et al., 2009) that leads to the stabilization of end-on kinetochore-microtubule attachments. Cdk1 downregulation due to cyclin A and B1 degradation might generate positive feedback loops that, in coordination with PEFs, further stabilize kinetochore-microtubule attachments (Collin et al., 2013; Kabeche...
and Compton, 2013; Mirkovic et al., 2015). While this eventually leads to SAC satisfaction after a significant mitotic delay in SMUGs, we propose that during normal mitosis this mechanism contributes to the stabilization of initial end-on kinetochore-microtubule attachments, before tension from opposing spindle forces is established during bi-orientation.
EXPERIMENTAL PROCEDURES

Quantification of Kinetochore-Microtubule Attachments

In order to distinguish the different types of kinetochore-microtubule attachments in SMUGs, we performed live-cell imaging in Drosophila S2 cells stably expressing GFP-α-tubulin/Cid-mCherry. Images were analyzed using Fiji (ImageJ) software through z stacks (0.5 μm). Kinetochore-microtubule attachments were quantified after tracing microtubule positioning in relation to the Cid signal (kinetochores). When microtubules passed by the Cid signal the attachment was considered as lateral. When microtubules ended at the kinetochore they were considered as end-on attachments. Since in SMUGs chromosomes do not align in the spindle equator, merotelic attachments were rarely observed and were distinguished as having long K-fibers coming from opposite poles that ended on the same kinetochore.

Measurement of Intra-kinetochore Stretch/Deformation

Drosophila S2 cells stably expressing Cid-mCherry/Ndc80-GFP (Maresca and Salmon, 2009) were used for intra-kinetochore stretch measurements in fixed (4% paraformaldehyde) material and for live-cell imaging (intra-kinetochore stretch measurements over time). Sub-pixel determination of fluorescent spot localization was performed using a home-written MATLAB script (Math-Works). A sequential refinement of the spot position starts with manual (mouse) selection of the kinetochore ensemble to be measured. A neighborhood region of interest (ROI) (11 × 11 pixels) is defined around each selected point, the boundary of which is used to estimate average background signal per pixel. This background value is subtracted, and the centroid is then calculated to allow recentering of the ROI. This first part of the script is meant as a coarse correction of the mouse-defined points. Before fitting a circular two-dimensional Gaussian function to each ROI intensity map, an empirical parameter of 1/2 was chosen as the fraction of (highest gray value) ROI pixels to be fed into the fitting procedure thus avoiding the bias induced by residual fluorescence of adjacent structures (e.g., defocused adjacent kinetochores). Fitting is performed using the least-squares fitting routine lsqcurvefit.

Statistical Analysis

Statistical analyses were performed using SigmaStat. Additional procedures are available in Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, three figures, and five movies and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.08.008.

AUTHOR CONTRIBUTIONS

D.D. performed and analyzed all the experiments; A.J.P. developed the algorithm to measure intra-kinetochore stretch on individual kinetochores; M.B. performed data analysis and designed experiments; T.J.M. provided reagents; H.M. performed data analysis, designed experiments, and supervised the work; D.D. and H.M. wrote the paper.

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Polar Ejection Forces Promote the Conversion from Lateral to End-on Kinetochore-Microtubule Attachments on Mono-oriented Chromosomes

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Figure S1. Single chromatids in SMUGs are laterally attached to spindle microtubules causing slow cyclin B1 degradation and a mitotic delay (related to Figure 1). (A) Live cell imaging of Drosophila S2 cells (control and Dup-depleted) stably expressing GFP-α-tubulin (red) and Cid-mCherry.
Dashed boxes illustrate examples of single kinetochores (lateral and merotelic attachments). (B) Immunoblot showing Dup depletion efficiency. Total cell protein extracts from Drosophila S2 cells stably expressing Dup-GFP were loaded into the wells. Dup depletion was monitored by using a rabbit anti-GFP antibody. A cell line stably expressing another GFP-tagged protein (Cyclin B1-GFP) was used as negative control. (C) Rabbit anti-Mad2 antibody was used to monitor Mad2 depletion. Mouse anti-tubulin was used to detect α-tubulin (loading control). Handwritten lines are from our annotations in the original film and represent the molecular weight (kDa). (D) Immunoblot showing the total amount of Mad2 in control and Dup-depleted cells. (E) Live cell imaging of control and Dup depleted cells stably expressing Cyclin B1-GFP (green) and mCherry-α-tubulin (red). (F) Cyclin B1 degradation profile in normal mitosis (black) (n=12 cells) and SMUGs (red) (n=4 cells) as indicated by fluorescence intensity quantification of Cyclin B1-GFP. The oscillatory decay pattern resulted from an artifact during image acquisition (periodic alterations in focal plane). Zero time point=NEB. Time is in min. Scale bar = 5 μm. Error bars represent standard deviation.
Figure S2. Unattached kinetochores in SMUGs have normal Mad2 and active Aurora B levels (related to Figure 1). (A, B) Immunofluorescence of Drosophila S2 control and Dup RNAi cells using an antibody against Mad2 (green) or pAuroraB T232 (green). Cid is shown in red and DNA (DAPI) in blue. (C) Mean Fluorescence Intensity of Mad2 levels normalized to Cid levels in control (n=207 kinetochores) and DupRNAi (n=160 kinetochores) depleted
Drosophila S2 cells. (D) Mean Fluorescence Intensity of p-T232 AuroraB levels normalized to Cid levels in control (n=37 kinetochores) and Dup RNAi (n=119 kinetochores) Drosophila S2 cells. There was no difference in the protein levels per kinetochore (normalized to Cid levels) between Dup RNAi and control cells. Scale bar = 5 μm. Error bars represent standard deviation. (ns = non-significant; a.u = arbitrary units)
Figure S3. Chromokinesin-mediated PEFs are required for timely SAC satisfaction in SMUGs (related to Figure 3). (A) Live cell imaging of H2B-
GFP (green) and mCherry-α-tubulin (red) Dup and Dup/Nod depleted Drosophila S2 cells treated with 40 μM Binucleine-2 immediately after NEB. (B) Live cell imaging of H2B-GFP (green) and mCherry-α-tubulin (red) Dup/Mad2 RNAi and Dup/Nod/Mad2 RNai Drosophila S2 cells. (C) Mitotic duration of Dup RNAi (t=111 ± 43 min, Mean ± SD, n=11 cells), Dup RNAi after Binucleine-2 addition (t=29.65 ± 4.31 min Mean ± SD, n=18), Dup/ Mad2 RNAi (t=22.1 ± 6.0 min, Mean ± SD, n=31 cells, p<0.001, Mann-Whitney Rank Sum test). (D) Mitotic duration of Dup/Nod RNAi, (t=208 ± 109 min, Mean ± SD, n=25 cells), Dup/Nod RNAi + Binuclein-2 (t= 26.71 ± 5.52 min, Mean ± SD, n=15), Dup/Nod/ Mad2 RNAi (t=24 ± 4.9, n=7, p<0.001, Mann-Whitney Rank Sum test) showing that the mitotic delay observed in chromokinesin-depleted SMUGs is due to inefficient satisfaction of the SAC. (E) Live cell imaging of Nod/Klp3A and Dup/Nod/Klp3A depleted Drosophila S2 cells stably expressing H2B-GFP (green) and mCherry-α-tubulin (red). Depletion of both Chromokinesins led to increased mitotic duration and chromosome alignment defects in control cells and SMUGs. Time=h:min. Scale bar = 5 μm. Error bars represent standard deviation.
SUPPLEMENTAL MOVIE LEGENDS:

**Movie S1 (related to Figure 1).** Top panels show live cell imaging of control and Dup-depleted Drosophila S2 cells stably expressing GFP-H2B (green) and mCherry-α-tubulin (red). Lower panels show live cell imaging of control and Dup-depleted Drosophila S2 cells stably expressing GFP-α-tubulin (red) and Cid-mCherry (green). Note the formation of lateral and merotelic attachments in SMUGs. Spinning-disk confocal images were acquired every 2 min (11 Z planes with 0.5 μm step size) using 100x objective and 1.5x tube lens (optivar) (75 nm/pixel sampling). Movies represent a maximum intensity projection of all stacks. Time=h:min.

**Movie S2 (related to Figure 1).** Live cell imaging of control and Dup-depleted Drosophila S2 cells stably expressing BubR1-mCherry (green) and GFP-α-tubulin (red). BubR1 signal vanishes prior to mitotic exit both in control cells and SMUGs. Spinning-disk confocal images were acquired every 2 min (11 Z planes with 0.5 μm step size) using 100x objective and 1.5x tube lens (optivar) (75 nm/pixel sampling). Movies represent a maximum intensity projection of all stacks. Time=h:min.

**Movie S3 (related to Figure 3).** Live cell imaging of control, Nod-depleted, Dup-depleted and Dup/Nod-depleted Drosophila S2 cells stably expressing GFP-H2B (green) and mCherry-α-tubulin (red). Nod depletion increased mitotic duration in both control and SMUGs, and caused mild chromosome alignment defects in control cells. Spinning-disk confocal images were
acquired every 2 min (11 Z planes with 0.5 μm step size) using 100x objective (112 nm/pixel sampling). Movies represent a maximum intensity projection of all stacks. Time=h:min.

**Movie S4 (related to Figure 3).** Live cell imaging of control and Dup-depleted Drosophila S2 cells stably expressing GFP-α-tubulin (red) and Nod-mCherry (green). Increased PEFs led to stabilization of kinetochore-microtubule attachments. Spinning-disk confocal images were acquired every 2 min (11 Z planes with 0.5 μm step size) using 100x objective and 1.5x tube lens (optivar) (75 nm/pixel sampling). Movies represent a maximum intensity projection of all stacks. Time=h:min.

**Movie S5 (related to Figure 4).** Live cell imaging of Klp61F/Dup and Klp61F/Dup/Nod-depleted Drosophila S2 cells stably expressing GFP-α-tubulin (red) and Cid-mCherry (green). Nod depletion increased the mitotic duration of Dup-depleted cells with monopolar spindles. Spinning-disk confocal images were acquired every 2 min (11 Z planes with 0.5 μm step size) using 100x objective and 1.5x tube lens (optivar) (75 nm/pixel sampling). Movies represent a maximum intensity projection of all stacks. Time=h:min.
SUPPLEMENTAL EXPERIMENTAL PROCEDURES:

Cell culture and drug treatments
Drosophila S2 cells were kept in Schneider’s medium (Gibco-BRL) containing 10% heat inactivated fetal bovine serum (FBS) (Invitrogen) at 25°C incubator. For live cell imaging, we used cell lines stably expressing fluorescent proteins: GFP-H2B/mCherry-α-tubulin (Afonso et al., 2014), GFP-α-tubulin/Cid-mCherry (Matos et al., 2009), Cyclin B1-GFP/mCherry-α-tubulin (Afonso et al., 2014), BubR1-mCherry/GFP-α-tubulin (Matos et al., 2009), GFP-α-tubulin/Nod-mCherry (Cane et al., 2013), and Cid-mCherry/Ndc80-GFP (Maresca and Salmon, 2009). For colchicine treatment, 200 μM Colchicine (Sigma-Aldrich) was added immediately after NEB both in control cells and SMUGs. Dup and Dup/Nod RNAi cells were treated with 40 μM Binucleine -2 (Sigma-Aldrich) after NEB to inhibit SAC response (Moutinho-Pereira et al., 2013).

RNA interference
S2 cells were plated in six-well plates (1x10^6 cells/well), and incubated for 2 h with Schneider’s medium without FBS containing 10 μg/ml of Dup, Nod or Mad2 dsRNA. For dsRNA synthesis, we used following primers: (Dup) forward: 5'-TAATACGACTCACTATAGGGGTCATAACGTGTGCATGG-3' and reverse: 5'-TAATACGACTCACTATAGGGGTCATAACGTGTGGATTCATGG-3' (Nod) forward 5'-TAATACGACTCACTATAGGGGACCTGGGTATTCTGCCTCG-3'
and reverse 5’-
TAATACGACTCCTATAGGGGATATGGATCCTGATGTGGGCC-3’;
(Klp3a) forward 5’-
TAATACGACTCCTATAGGGGATATGGATCCTGATGTGGGCC-3’; reverse
5’- TAATACGACTCCTATAGGGGATATGGATCCTGATGTGGGCC-3’;
(Mad2) forward 5’-TAATACGACTCCTATAGGGGATATGGATCCTGATGTGGGCC-3’, reverse 5’-TAATACGACTCCTATAGGGGATATGGATCCTGATGTGGGCC-3’ (Orr et al., 2007); (Klp61F) forward 5’-
TAATACGACTCCTATAGGGGATATGGATCCTGATGTGGGCC-3’, reverse
5’-TAATACGACTCCTATAGGGGATATGGATCCTGATGTGGGCC-3’. After 2 h, normal Schneider’s medium (Gibco-BRL) with 10% FBS was added and cells were kept for 96 h at 25°C for efficient knockdown (Maiato et al., 2003).

**Cell transfection**

Control Drosophila S2 cells or Dup depleted stably expressing GFP-H2B/mCherry-α-tubulin were transiently transfected with non-degradable Cyclin B1 (Δ90CyclinB1) (Afonso et al., 2014) using Effectene Transfection Reagent (Qiagen) according to manufacturer’s protocol. Similarly, parental Drosophila S2 cells were also stably transfected with Dup-Dest-13-GFP plasmid.

**Immunofluorescence**

Control cells and SMUGs were seeded on Concanavalin A (Merk Milipore) treated coverslips. Fixation was performed with 4% paraformaldehyde, followed by extraction with 0.3% Triton in PBS for 10 min. Cells were washed
three times with PBS + 0.05% Tween, and blocked in 10% FBS for 1h, followed by primary antibody incubation for 1h at room temperature. After primary antibody incubation, coverslips were washed three times with PBS + 0.05% Tween and incubated for 45min at room temperature with the secondary antibodies (Alexa 488, Alexa 568, Alexa 647, Invitrogen 1:1000 dilutions) in blocking solution. After washing, cells were incubated 5 min with DAPI (Sigma, 1 μg/ml), washed with PBS and sealed on slides using mounting media (20 mM Tris pH8, 0.5 N-propyl gallate, 90% glycerol). Primary antibodies used were: mouse anti-α-tubulin (1:2000; B-512 clone, Sigma); rat anti-Cid (1:2000), rabbit-anti-Mad2 and rabbit-anti-Mad1 (1:1000) (kind gifts from Claudio Sunkel, IBMC, University of Porto, Portugal) (Conde et al., 2013), rabbit anti-pKNL1 (1:1000) (Welburn et al., 2010) (kind gift from Iain Cheeseman, Whitehead Institute, MIT, Boston), rabbit anti-pT232 AuroraB (Rockland Immunnochemicals). For image analysis and acquisition we used a Zeiss AxioObserver Z1 wide-field microscope equipped with a plan-apochromat (1.46 NA 100x) DIC objective and a cooled CCD (Hamamatsu Orca R2). Images were deconvolved using Autoquant X (Media Cybernetics) and processed for publication with Fiji (ImageJ) and Adobe Photoshop CS4 (Adobe Systems).

**Live cell imaging**

Drosophila S2 cells were plated on Concanavalin A coated (0.25 mg/ml) MatTek dishes (MatTekCorporation) 2-3 h prior to live cell imaging. Live imaging of GFP-α-tubulin/Cid-mCherry, H2B-GFP/mCherry-α-tubulin, CyclinB1-GFP/mCherry-α-tubulin, BubR1-mCherry/GFP-α-tubulin, GFP-α-
tubulin/Nod-mCherry was performed on a temperature-controlled Nikon TE2000 microscope equipped at the camera port with a modified Yokogawa CSU-X1 spinning-disc head (Solamere Technology), an ASI FW-1000 filter-wheel and an Andor iXon+ DU-897 EM-CCD. The excitation optics is composed of two sapphire lasers at 488 nm and 561 nm (Coherent) which are shuttered by an acousto-optic tunable filter (Gooche&Housego, model R64040-150) and injected into the Yokogawa head via a polarization-maintaining single-mode optical fiber (OZ optics). Sample position is controlled by a SCAN-IM stage (Marzhauser) and a 541.ZSL piezo (Physik Instrumente). The objective is an oil-immersion 100x 1.4 NA Plan-Apo DIC CFI (Nikon, VC series), yielding an overall (including the pinhole-imaging lens) 112 nm/pixel sampling. A 1.5x tube lens (optivar) was also used (75 nm/pixel sampling). A 2 min time-lapse interval and 0.5 μm step image stack was used in all acquisitions, except for intra-kinetochore stretch measurements where it was used a 15 min time interval and 0.22 μm step image stack. The system was controlled by NIS-Elements via a DAC board (National Instruments, PCI-6733).

**Immunoblotting**

Immunoblotting was used to confirm protein depletion. After boiling protein extracts for 5 min in SDS sample buffer, samples were run on SDS-PAGE and transferred to nitrocellulose membranes. Blots containing proteins were incubated with primary antibodies: mouse anti-α-tubulin (1:2000; B-512 clone, Sigma), rabbit anti-GFP (kindly provided by Frederico Silva, IBMC, University
of Porto, Portugal), rabbit-anti-Mad2 (1:1000) (kind gift from Claudio Sunkel, IBMC, University of Porto, Portugal) (Conde et al., 2013).

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