Chromatids segregate without centrosomes during *Caenorhabditis elegans* mitosis in a Ran- and CLASP-dependent manner

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ABSTRACT **During mitosis, chromosomes are connected to a microtubule-based spindle. Current models propose that displacement of the spindle poles and/or the activity of kinetochore microtubules generate mechanical forces that segregate sister chromatids. Using laser destruction of the centrosomes during *Caenorhabditis elegans* mitosis, we show that neither of these mechanisms is necessary to achieve proper chromatid segregation. Our results strongly suggest that an outward force generated by the spindle midzone, independently of centrosomes, is sufficient to segregate chromosomes in mitotic cells. Using mutant and RNAi analysis, we show that the microtubule-bundling protein SPD-1/MAP-65 and BMK-1/kinesin-5 act as a brake opposing the force generated by the spindle midzone. Conversely, we identify a novel role for two microtubule-growth and nucleation agents, Ran and CLASP, in the establishment of the centrosome-independent force during anaphase. Their involvement raises the interesting possibility that microtubule polymerization of midzone microtubules is continuously required to sustain chromosome segregation during mitosis.**

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INTRODUCTION

The mitotic spindle is a microtubule network, which consists of kinetochore microtubules, to which chromosomes are attached via their kinetochores, and nonkinetochore microtubules. The nonkinetochore microtubules can emanate from the spindle poles or, for instance, be nucleated directly around DNA (Heald *et al.*, 1996, 1997; Walczak *et al.*, 1998). The mechanisms responsible for chromosome capture and alignment have been extensively explored. In contrast, less is known about the different mechanical forces generated by the spindle to allow chromatid segregation during anaphase and the molecules involved in this process (Goshima and Scholey, 2010).

In most eukaryotic cells, chromatids can be displaced on the mitotic spindle in two nonexclusive ways. First, in spindles containing centrosomes (or spindle pole bodies [SPBs]), centrosome movements drive chromatid separation in a process called anaphase B. This can be achieved by traction forces acting on the astral microtubules emanating from the poles (Carminati and Stearns, 1997; Grill *et al.*, 2001). Microtubules emanating from each centrosome or SPB can also cross in the spindle midzone and slide on each other to push the spindle poles apart (Brust-Mascher *et al.*, 2004; Khodjakov *et al.*, 2004; Tolić-Nørrelykke *et al.*, 2004). During anaphase A, chromatids are pulled toward the spindle poles by the action of kinetochore microtubules (Nicklas, 1989). This is achieved by either a "Pac-Man" mechanism of minus-end microtubule depolymerization at the poles or a direct pulling on the kinetochores (Mitchison and Salmon, 1992; Skibbens *et al.*, 1993; Zhai *et al.*, 1995; Desai *et al.*, 1998, 1999; Rogers *et al.*, 2004). Recently it was shown that sliding of kinetochore microtubules on adjacent microtubules is another mechanism leading to chromosome displacement toward the pole during anaphase A (Elting *et al.*, 2014; Sikirzhytski *et al.*, 2014). All of

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Abbreviations used: GAP, GTPase-activating protein; GEF, guanine nucleotide exchange factor; IR, infrared; NEDB, nuclear envelope breakdown; OICD, optically induced centrosome disruption.

these movements are independent of the presence of centrosomes or SPBs. Consequently, acentrosomal spindles such as female meiotic spindles and those found in plant cells or mouse early embryos are capable of sustaining chromatid segregation (Delattre and Gönczy, 2004; Zhang and Dawe, 2011). Similarly, after experimental removal of centrosomes from centrosome-containing cells, such as human or fly cells, anaphase A is sufficient to allow chromatid segregation (Khodjakov *et al.*, 2000; Basto *et al.*, 2006). However, kinetochores are not needed for chromatid separation during *Caenorhabditis elegans* acentrosomal female meiosis (Dumont *et al.*, 2010), and DNA-coated beads lacking kinetochores are also able to move on artificial acentrosomal spindles (Deng *et al.*, 2009). Thus, in these atypical spindles, a mechanical force, independent of both centrosomal movements and the activity of kinetochore microtubules, can sustain chromatid segregation. In this work, we asked whether chromatid segregation can also occur independently of centrosomes and of the traction exerted by kinetochore microtubules during mitosis.

To address this question, we analyzed the mitotic spindle of one-cell *C. elegans* embryos. In these cells, laser ablations of the central spindle performed at the onset of anaphase revealed that the spindle poles are subjected to robust unbalanced cortical pulling forces acting on astral microtubules. These forces control the asymmetric positioning of the mitotic spindle and contribute to chromatid separation (Grill *et al.*, 2001; Labbe *et al.*, 2004; Pecreaux *et al.*, 2006). In contrast, no poleward flux of kinetochore microtubules has been observed during anaphase in the one-cell *C. elegans* embryo (Labbe *et al.*, 2004). Moreover, the distance between centrosomes and chromatids is almost constant throughout anaphase, strongly suggesting that the movement of chromatids on kinetochore microtubules (i.e., anaphase A) is unlikely to play a significant role in chromatid segregation in these cells (Oegema *et al.*, 2001). Overall, anaphase B dominates during *C. elegans* mitosis.

Of interest, reducing the pulling forces by depleting the proteins involved in cortical force generation does not prevent chromatid separation in *C. elegans* embryos, although their separation is less efficient than in wild-type cells (Colombo *et al.*, 2003; Gotta *et al.*, 2003; Srinivasan *et al.*, 2003). This suggests either that cortical pulling forces are not fully abolished in the mutants or by RNA interference (RNAi) treatments or that an outward force generated by the spindle pushes on the centrosomes. Alternatively, chromatid segregation could occur independently of centrosomes in the one-cell *C. elegans* embryo. To discriminate between these possibilities and further explore the existence of a mechanical force independent of both Anaphase A and centrosomes, we performed a physical destruction of centrosomes during *C. elegans* mitosis.

RESULTS

Chromatids segregate in the absence of centrosomes during *C. elegans* anaphase

In *C. elegans* one-cell embryos, the metaphase spindle sets up in the center of the cell. During anaphase, the spindle simultaneously elongates, oscillates, and becomes posteriorly displaced (Figure 1A and Supplemental Video S1). To analyze precisely chromosome segregation in the absence of cortical pulling forces during *C. elegans* mitosis, we abolished the source of these forces by destroying centrosomes with a laser microbeam. We performed optically induced centrosome disruption (OICD) during the first cell cycle using either an infrared (IR) or a pulsed ultraviolet (UV) laser (Figure 1B and Supplemental Videos S2 and S3; Grill *et al.*, 2003; Labbe *et al.*, 2004). In this study, we quantified the effects of laser ablation mostly after UV treatments. We used a transgenic strain expressing histone H2B and

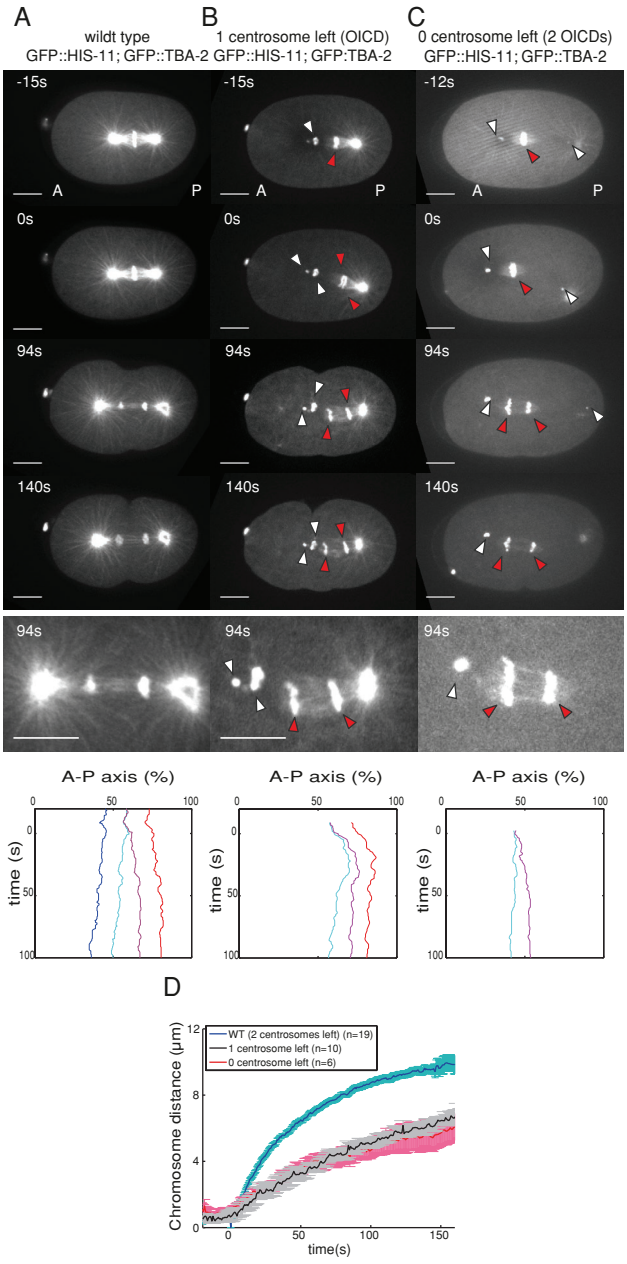

FIGURE 1: Chromatids separate in the absence of centrosomes. (A–C) Snapshots and kymographs of GFP::tubulin; GFP::histone embryos in wild-type controls (A), after laser destruction (OICD) of the anterior centrosome (B), and after OICD of both centrosomes (C). Red and white arrowheads point to chromatids and the plasma generated by the UV laser, respectively. In this and all subsequent figures, the anterior pole of the cell is to the left. Kymographs display centrosome (blue and red) and chromatid (cyan and magenta) trajectories as percentage of total cell length. (D) Average curves of the chromatid-to-chromatid distance in micrometers over time for wild-type embryos (blue) after OICD of one centrosome (black) or double OICD (red). Scale bar, 10 µm. $t = 0$ s: chromatid separation onset. Errors bars, SD.

α-tubulin fused to green fluorescent protein (GFP). This allowed us to precisely target centrosomes and observe microtubules after centrosome destruction. This also allowed us to use chromosome condensation and alignment as indicators of mitosis progression.

First, we ablated one centrosome 10–30 s before the onset of chromatid separation, which corresponded to ~120 s after nuclear

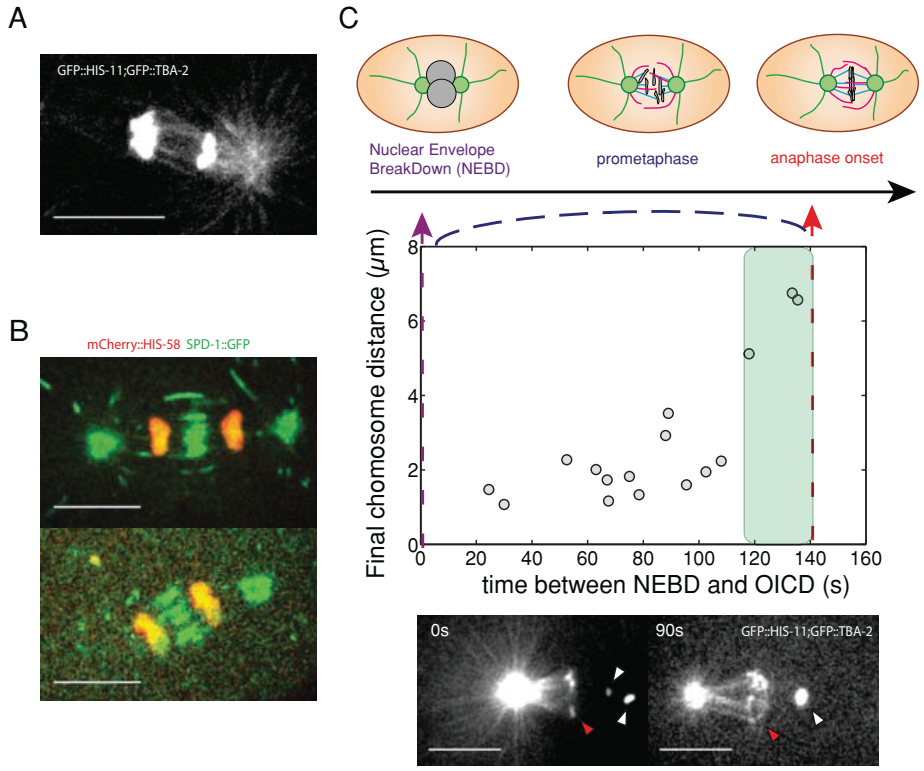

FIGURE 2: Midzone microtubules assemble after OICD. (A) Confocal image of a GFP::tubulin; GFP::histone embryo after IR laser destruction of the anterior centrosome. Exposure time is 6 s. (B) Snapshots of embryos coexpressing SPD-1::GFP and mCherry:HIS-58 in wild-type control (top) and after destruction of the anterior centrosome with a UV laser (bottom). Scale bar, 10 μm. (C) Chromatid-to-chromatid distance in micrometers measured 370 s after NEBD (this time was chosen because it corresponds to the end of mitosis) relative to the time elapsed between NEBD and centrosome ablation. The green area represents the time window used in the rest of our study. Bottom, snapshots of a one-cell stage GFP::tubulin; GFP::histone embryo for which the posterior centrosome was UV irradiated early during mitosis. $t = 0$ s corresponds to the time of OICD. White and red arrowheads point to the plasma and the chromosomes, respectively.

envelope breakdown (NEBD). After OICD of one centrosome, we observed a rapid movement of both sets of chromatids together with the intact centrosome toward the opposite pole of the cell. This movement is due to the release of cortical pulling forces from one side of the cell, while the intact centrosome is still being pulled, and demonstrates the efficacy of centrosome ablation (see *Materials and Methods*, Figure 1B, and Supplemental Figure S1, A–C). After this initial movement of the spindle, we found that the "free" chromatids (those on the same side of the cell as the ablated centrosome) rapidly separated from their sister chromatids (Figure 1B). We found that the extent and rate of chromatid separation were lower after centrosome ablation than in intact cells (Figure 1D and Supplemental Figure S1D). Nevertheless, 2 min after OICD, corresponding to the end of anaphase in intact cells, chromatids had separated ~6 μm in cells with one centrosome ablated compared with 10 μm in intact cells (Figure 1D). Previous experiments demonstrated that incomplete OICD can generate small aster fragments (Grill *et al.*, 2003). These small fragments could potentially reestablish contacts between the cortex and the spindle and contribute to chromatid segregation. To exclude this possibility, we performed OICD in embryos in which the force-generating complex was depleted. We also performed a simultaneous OICD of both centrosomes in wild-type embryos. We reasoned that in both conditions, pulling forces exerted by the remaining small aster fragments should be drastically reduced. First, we performed OICD in *lin-5(RNAi)*–treated embryos. Inactivation of *lin-5*, one component of the force-generating complex, fully abolishes cortical pulling forces (Nguyen-Ngoc *et al.*, 2007). As expected, we found that in intact *lin-5(RNAi)*–treated embryos, the mitotic spindle remained in the center of the cell and failed to oscillate, and its elongation was limited compared with wild-type cells. Despite these effects, chromosome segregation was similar in both *lin-5(RNAi)*–treated embryos and wild-type controls after OICD (Supplemental Figure S1E). Second, we destroyed both centrosomes simultaneously 10–30 s before the onset of chromatid separation in wild-type embryos (Figure 1C and Supplemental Video S4). We found that the speed of chromatid separation was similar after single or double OICD (Figure 1D and Supplemental Figure S1D). This demonstrates that after OICD, chromatid segregation is not due to a remnant of cortical pulling forces exerted on both sides of the spindle. The destruction of both centrosomes also ruled out the possibility that microtubules emanating from the intact centrosome could push on the "free" chromatid mass. Because we found the same rate of chromatid segregation after single or double OICD, we also conclude that an isolated centrosome cannot contribute to chromosome segregation. Of importance, although chromatids appeared less organized at the end of mitosis after single or double OICD compared with intact cells, they did not form chromatin bridges and did not lag on the spindle (Figure 1, B and C, and Supplemental Videos S2–S4). Moreover, we did not observe karyomeres associated with the reforming nuclei in daughter cells. This strongly suggests that chromatids are correctly segregated in the absence of centrosomes. Overall these experiments demonstrate that although centrosome-dependent forces contribute to chromatid separation, they are dispensable for chromatid separation during anaphase in the one-cell *C. elegans* embryo. In *C. elegans* embryos, chromatids are not displaced on kinetochore microtubules (Oegema *et al.*, 2001; Labbe *et al.*, 2004). Our results therefore strongly suggest that the spindle is able to generate an outward force to segregate chromatids independently of centrosomes.

Midzone microtubules assemble at the onset of anaphase after centrosome destruction

Despite the strong photobleaching of fluorescence signals caused by the laser ablation of centrosomes, we could still detect microtubules between the separating chromatids after OICD (Figure 2A and Supplemental Video S2). To ask whether these microtubules might correspond to stabilized antiparallel microtubules, we performed single OICD in a transgenic strain expressing SPD-1 fused to GFP. SPD-1 is the *C. elegans* homologue of MAP-65/PRC1/Ase1, a conserved cross-linker of antiparallel microtubules that is recruited to the central spindle during anaphase (Mollinari, 2002; Verbrugghe and White, 2004; Braun *et al.*, 2011). We found that after OICD, the SPD-1::GFP signal still accumulated in between separating chromatids, as

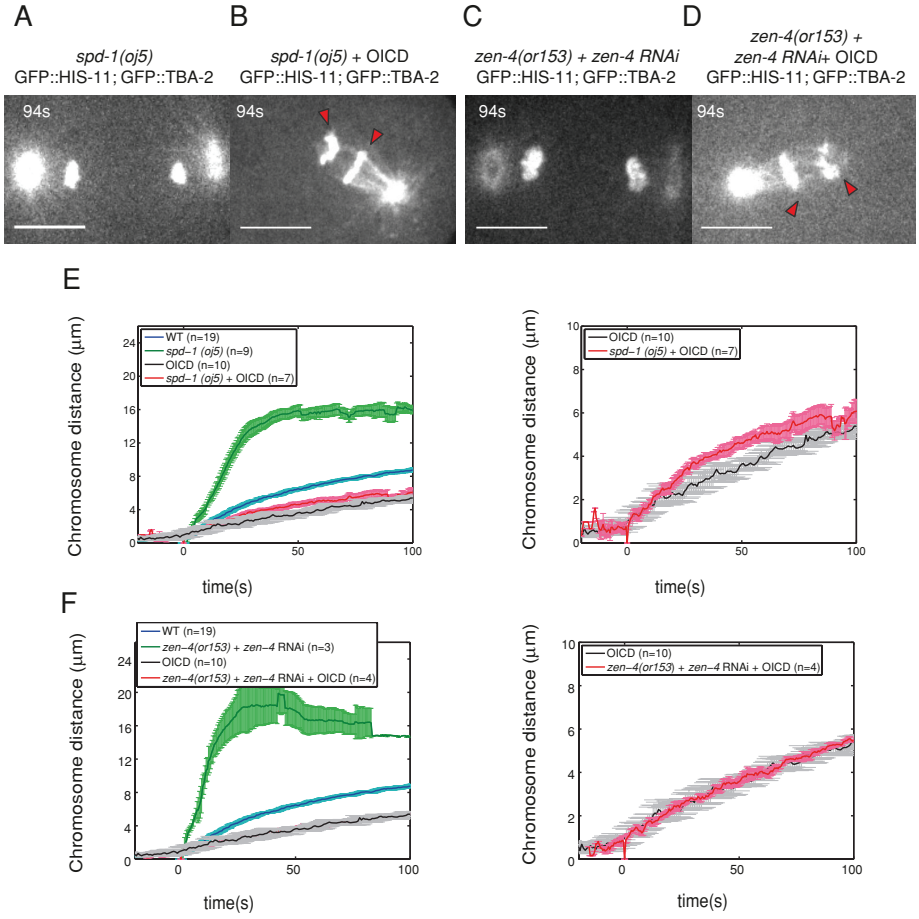

FIGURE 3: Role of SPD-1 and ZEN-4 on the force generated independently of centrosomes. (A–D) Snapshots of GFP::tubulin; GFP::histone embryos carrying the *spd-1(oj5)* allele in intact cells (A) or after OICD of the anterior centrosome (B), or carrying the *zen-4(or153)* allele and treated with *zen-4(RNAi)* with intact centrosomes (C) or after OICD of the posterior centrosome (D). The red arrowheads point to DNA. (E, F) Average curves of the chromatid-to-chromatid distance in micrometers over time in intact cells or after OICD for wild-type (blue and black curves, respectively) and mutants cells (green and red curves, respectively). Right, only the curves corresponding to the OICD experiments are shown. *spd-1(oj5)* mutants are shown in E, and *zen-4(or153)* is shown in F. Scale bar, 10 µm. $t = 0$ s: chromatid separation onset. Errors bars, SD.

in intact wild-type cells (Figure 2B and Supplemental Video S5). This suggests that despite the absence of one centrosome, cross-linked antiparallel midzone microtubules are still formed in the central spindle of *C. elegans* embryos, as previously observed after centrosome destruction in other cell types (Khodjakov *et al.*, 2004).

We noticed that performing OICD earlier during mitosis, at 20–100 s after NEBD, prevented chromatid segregation (Figure 2C and Supplemental Video S6). Therefore, although centrosomes are dispensable for chromatid segregation during anaphase, they are necessary at the earlier steps of mitosis. We hypothesize that centrosomes are required early to correctly organize microtubules around the chromosomes and that this microtubule organization is later needed for chromosome segregation, independently of centrosomes.

We next aimed to identify microtubule-associated proteins that play a role in the force generated by the spindle independently of centrosomes. Because chromosome segregation is similar in the absence of one or two centrosomes, we analyzed the consequence of a single OICD in *C. elegans* mutant embryos or embryos treated with RNAi against candidate genes. OICD was performed at ~120 s after NEBD, corresponding to 20 s before the onset of chromatid segregation, on embryos expressing α-tubulin and histone H2B fused to GFP, allowing us to measure chromatid movements.

SPD-1 acts as a brake to oppose the force generated independently of centrosomes

As described, we found that SPD-1 is enriched in the spindle midzone after OICD in wild-type embryos (Figure 2B). We hypothesized that SPD-1 could be needed to generate an outward pushing force by stabilizing the antiparallel array of midzone microtubules, on which molecular motors can walk (Fu *et al.*, 2009; Khmelinskii *et al.*, 2009). Conversely, SPD-1 could act as a brake by increasing microtubule bundling in the spindle midzone, as previously reported for its yeast orthologue, Ase1 (Braun *et al.*, 2011; Lansky *et al.*, 2015). Inactivation of *spd-1* in intact *C. elegans* embryos does not affect the formation of the metaphase spindle but leads to spindle breakage and very rapid separation of chromatids during anaphase due to the strength of cortical forces pulling on both centrosomes (Figure 3, A and E, and Supplemental Figure S2A; Verbrugghe and White, 2004). Of importance, when we treated embryos with RNAi against *spd-1* and *gpr-1/2*, a component of the cortical force-generating complex (Colombo *et al.*, 2003), the spindle did not break, and we could detect midzone microtubules (Supplemental Figure S2F). Moreover, these microtubules were decorated with the central spindle component kinesin-6/ZEN-4 (Supplemental Figure S2F; Mishima *et al.*, 2002). Therefore, although SPD-1 is involved in the stabilization of the spindle midzone upon external pulling forces, it is not required for its formation. To test the role of SPD-1 in the centrosome-independent force, we performed OICD in embryos carrying a null allele of *spd-1*. As expected, we found that destruction of one centrosome in these embryos 10–30 s before the onset of chromosome separation did not prevent the formation of microtubules between the separating chromatids (Figure 3B). However, we observed a larger extent of chromatid separation at later stages compared with OICD performed in wild-type cells (Figure 3, B and E, and Supplemental Figure S2, B and E). Therefore, despite the absence of a tension exerted by cortical pulling forces on each spindle pole, SPD-1 is required to restrain chromosome segregation. We concluded that SPD-1 acts as a brake to oppose the force generated by the spindle independently of centrosomes in *C. elegans* embryos.

In the absence of centrosomes, ZEN-4 is not involved in chromatid segregation

The kinesin-6/MKLP1 ortholog ZEN-4 is recruited to the spindle midzone during anaphase and interacts with the Rho GTPase-activating protein (GAP) CYK-4 to form the highly conserved Centralspindin complex, which stabilizes the spindle midzone. Centralspindlin also activates the contractility of actin and controls cytokinesis during meiosis and mitosis (Mishima *et al.*, 2002; Hutterer *et al.*, 2009).

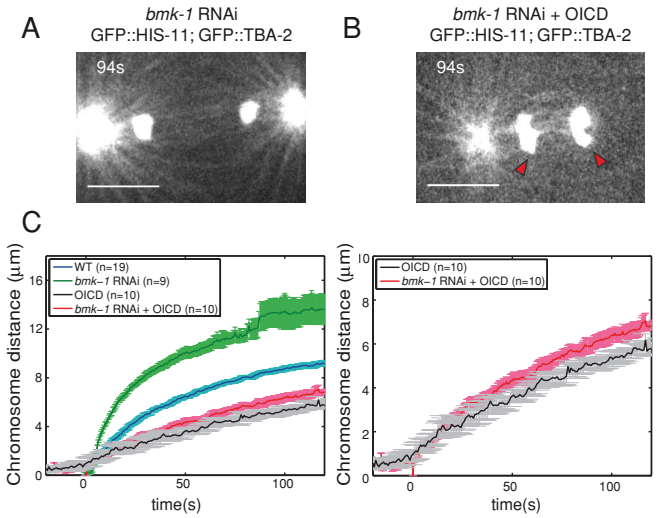

FIGURE 4: BMK-1 acts as a brake to oppose the force generated independently of centrosomes. (A, B) Snapshots of GFP::tubulin; GFP::histone in a *bmk-1(RNAi)* embryo with intact centrosomes (A) or after OICD of the posterior centrosome (B). Red arrowheads point to the chromatids. (C) Average curves of the chromatid-to-chromatid distance in micrometers over time in intact cells or after OICD for wild-type (blue and black curves, respectively) and *bmk-1(RNAi)* embryos (green and red curves, respectively). Right, only the curves corresponding to the OICD experiments are shown. Scale bar, 10 µm. $t = 0$ s: chromatid separation onset. Errors bars, SD.

Inactivation of Centralspindlin does not prevent the formation of the metaphase spindle but also leads to spindle breakage during anaphase in *C. elegans* embryos (Figure 3, C and F, and Supplemental Figure S2C; Mishima *et al.*, 2002). However, after inactivation of *zen-4* and *gpr-1/2* by RNAi, the spindle remained intact during anaphase, and we observed an accumulation of SPD-1 on the spindle midzone (Supplemental Figure S2G). This result strongly suggests that although ZEN-4 is required for the stabilization of the spindle midzone in response to external forces, it is not involved in the formation of the antiparallel array of microtubules. We asked whether the motor activity of ZEN-4 could play a role in the mechanical force generated independently of centrosomes during *C. elegans* anaphase. To this end, we performed OICD of one centrosome in the absence of ZEN-4. To achieve complete inactivation of ZEN-4, we combined an RNAi treatment of *zen-4* in mutant embryos carrying a null allele of *zen-4* mutants, as previously described (Severson *et al.*, 2000; Lewellyn *et al.*, 2011). When OICD was performed in these embryos, we still observed microtubules between the separating chromatids. Moreover, we observed a normal segregation of chromatid compared with OICD performed in wild-type cells (Figure 3, D and F, and Supplemental Figure S2, D and E). This result suggests that the kinesin ZEN-4 does not contribute to the force generated by the spindle independently of centrosomes.

BMK-1 acts as a brake against the force generated independently of centrosomes

In most cellular systems, kinesin-5 is the main molecular motor generating an outward pushing force during anaphase through the sliding of antiparallel microtubules in the midzone (Sharp *et al.*, 1999; Ferenz *et al.*, 2010) Unexpectedly, in *C. elegans* embryos, inactivation of the sole kinesin-5 homologue, *bmk-1*, leads to faster centrosome separation than with wild-type embryos. It has therefore been proposed that BMK-1 acts as a brake against the cortical forces pulling on the centrosomes during *C. elegans* mitosis (Figure 4, A and C, and Supplemental Figure S3A; Saunders *et al.*, 2007). We asked whether BMK-1 also opposes the pushing force generated by the spindle in the absence of centrosomes. To this end, we performed OICD in *bmk-1(RNAi)* embryos. We found that chromatids segregated to a larger extent in the absence of BMK-1 than with wild-type controls (Figure 4, B and C, and Supplemental Figure S3, B and C). We conclude that the divergent *C. elegans* kinesin-5 acts as a brake to oppose the force generated by the spindle independently of centrosomes.

CLASP is essential to establish a force in the absence of centrosomes

CLASP is a microtubule-stabilizing factor that promotes microtubule rescue and growth. In all organisms analyzed, it stabilizes kinetochore microtubules in the early stages of mitosis (Hannak, 2006; Al-Bassam *et al.*, 2010). Of interest, the *C. elegans* CLASP orthologue CLS-2 is required for proper chromosome separation during female acentrosomal meiosis, whereas kinetochores are dispensable (Dumont *et al.*, 2010). We therefore tested the requirement of CLS-2 during centrosome-independent chromatid separation in mitosis. Because of its role in kinetochore microtubule dynamics, disruption of *cls-2* affects chromosome alignment during metaphase in *C. elegans* embryos (Cheeseman *et al.*, 2005; Espiritu *et al.*, 2012). This prevented us from testing its role in subsequent stages of the cell cycle (Figure 5A and Supplemental Figure S4A). However, reducing the effect of cortical pulling forces in *cls-2(RNAi)* embryos restored chromosome alignment, chromosome biorientation, and partial chromatid separation at anaphase (Cheeseman *et al.*, 2005).

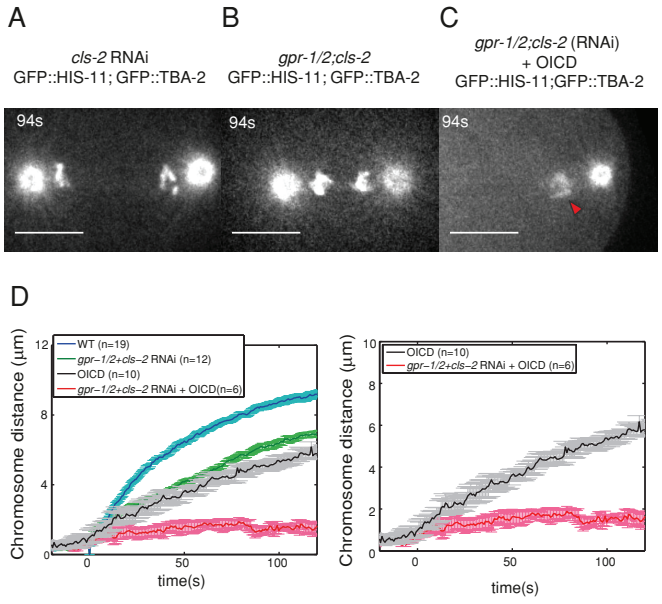

FIGURE 5: CLASP is essential for chromatid separation in the absence of centrosomes. (A–C) Snapshots of GFP::tubulin; GFP::histone in a *cls-2(RNAi)* embryo (A), a *gpr-1/2;cls-2(RNAi)* embryo with intact centrosomes (B), or a *gpr-1/2;cls-2(RNAi)* embryo after OICD of the anterior centrosome (C). The red arrowhead points to the unsegregated chromatids. (D) Average curves of the chromatid-to-chromatid distance in micrometers over time in intact cells or after OICD for wild-type (blue and black curves, respectively) and *cls-2;gpr-1/2(RNAi)* embryos (green and red curves, respectively). Right, only the curves corresponding to the OICD experiments are shown. Scale bar, 10 µm. $t = 0$ s: chromatid separation onset. Errors bars, SD.

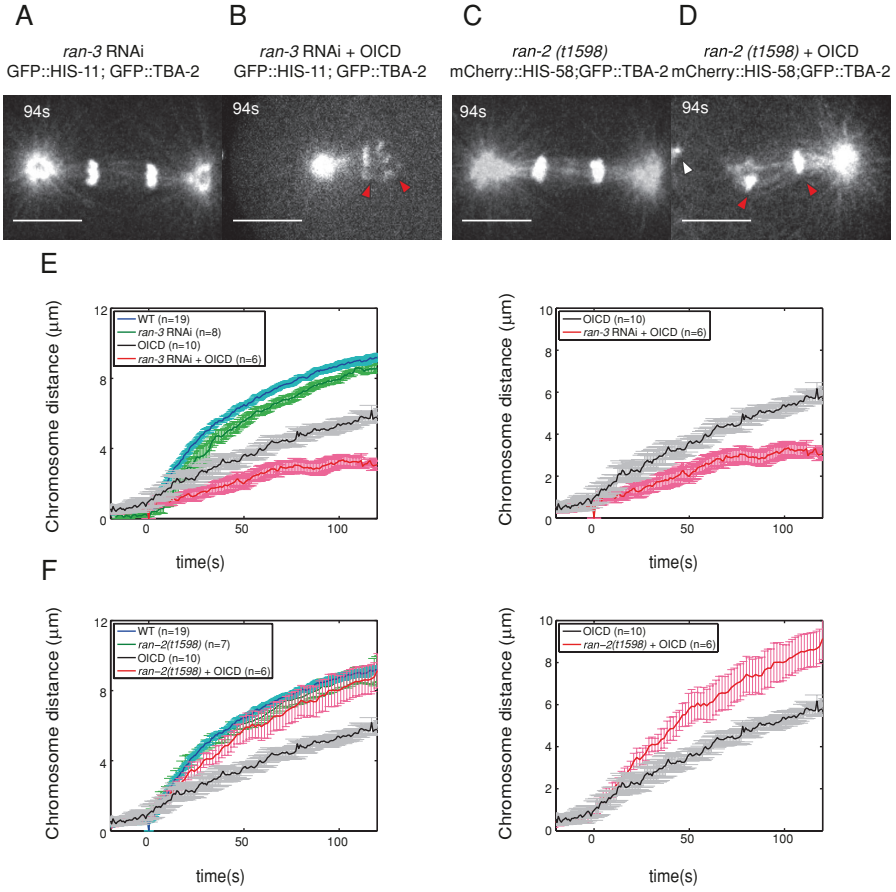

FIGURE 6: RanGTP promotes chromatid separation in the absence of centrosomes. (A–D) Snapshots of GFP::tubulin; GFP::histone in an *ran-3(RNAi)* embryo with intact centrosomes (A) or after OICD of the posterior centrosome (B), and in a *ran-2(t1598)* homozygous mutant with intact centrosomes (C) or after OICD of the anterior centrosome (D). Red arrowheads point to the chromatids. (E, F) Average curves of the chromatid-to-chromatid distance in micrometers over time in intact cells or after OICD for wild-type (blue and black curves, respectively) and mutant or RNAi-treated embryos (green and red curves, respectively). Right, only the curves corresponding to the OICD experiments are shown. *ran-3 (RNAi)* embryos are shown in E, and *ran-2(t1598)* is shown in F. Scale bar, 10 µm. $t = 0$ s: chromatid separation onset. Errors bars, SD.

maintenance of the spindle midzone during mitosis.

RanGTP promotes the force generated independently of centrosomes

Finally, we tested the role of the small GTPase Ran/RAN-1 during *C. elegans* anaphase. Inhibition of Ran affects spindle assembly in many different species through the reduction of microtubule density around the DNA (Carazo-Salas *et al.*, 1999; Kalab *et al.*, 1999; Ohba *et al.*, 1999; Wilde and Zheng, 1999; Zhang *et al.*, 1999). Because of this early effect on spindle formation, it has been difficult to address the role of Ran in the subsequent steps of the cell cycle in vivo. Therefore whether Ran is also required for anaphase spindle elongation remains unclear (Yokoyama *et al.*, 2009). To address the role of Ran during anaphase in the absence of centrosomes, we partially inactivated Ran guanine nucleotide exchange factor (GEF) RCC-1/RAN-3 or the RanGAP, RAN-2. On RanGEF inactivation, Ran remains in its inactive RanGDP-bound form. In *ran-3(RNAi)*–treated *C. elegans* embryos, RAN-3 is presumably only partially inactivated. As a consequence, although centrosomes first detach from the pronuclei in prometaphase, they eventually build a bipolar metaphase spindle, and anaphase proceeds (Askjaer *et al.*, 2002; Figure 6, A and E). We found that chromatid segregation was reduced after OICD in *ran-3(RNAi)*–treated embryos compared with OICD performed in wild-type cells (Figure 6, B and E, and Supplemental Figure S5A). This result shows that a partial reduction of RanGTP is sufficient to affect the segregation of chromatids independently of centrosomes during anaphase.

Therefore, to test the role of CLASP during anaphase, we treated embryos with RNAi against both *cls-2* and one component of the cortical force generator complex, *gpr-1/2,* as previously described (Cheeseman *et al.*, 2005; Figure 5, B and D, and Supplemental Figure S4B). The efficacy of the double gene inactivation was easily assessed by the presence of normal chromosome alignment on the metaphase plate (Supplemental Figure S4B). We then performed OICD on *gpr-1/2;cls-2 (RNAi)* embryos once the chromatids were properly aligned on the metaphase plate, ~20 s before they started to separate. We found that destruction of one centrosome in this context completely prevented chromatid segregation (Figure 5, C and D, and Supplemental Figure S4, C and D). This demonstrates that CLASP is necessary to allow chromosome segregation in the absence of centrosomes. Our result also strongly suggests that the separation of chromatids observed in *gpr-1/2;cls-2 (RNAi)* embryos with intact centrosomes is due to a remnant of cortical pulling forces due to partial inactivation of *gpr-1/2* by RNAi. Of interest, in *gpr-1/2;cls-2 (RNAi)* embryos harboring intact centrosomes, we could not detect any microtubules in between the separating chromatids by indirect immunofluorescence (Supplemental Figure S4E). Overall, similar to its role during *C. elegans* acentriolar meiosis (Dumont *et al.*, 2010), CLASP could be an essential player for the formation or

We next analyzed the consequences of having an excess of the active RanGTP-bound form on chromatid segregation by inactivating the *C. elegans* RanGAP RAN-2. Following RanGAP inactivation, Ran remains in its active, RanGTP-bound form. However, full inactivation of *ran-2* by RNAi prevents spindle formation in *C. elegans* embryos (Askjaer *et al.*, 2002). Therefore we characterized a mutant strain carrying a weak allele of *ran-2*, in which the mitotic spindle eventually forms (Gönczy *et al.*, 1999). We found that embryos from heterozygous *ran-2(t1598)* mutants displayed wild-type phenotypes of chromatid and centrosome segregation during mitosis (Figure 6C and Supplemental Figure S5, B and C). Embryos from homozygous *ran-2(t1598)* mutants displayed two categories of phenotypes. In the more severe cases, although a mitotic spindle formed, chromosomes failed to align and segregate during mitosis (Supplemental Figure S5D). In the other category of embryos, a first attempt to form a metaphase plate failed, and the centrosomes were separated from the chromosomes. However, the centrosomes traveled back toward the chromosomes in a second attempt, and a metaphase plate was eventually formed (Supplemental Figure S5E). In these embryos, the rate of chromatid segregation was similar to the rate observed in wild-type embryos during anaphase (Supplemental Figure S5C). However, the

distance between the centrosomes was slightly larger in embryos from homozygous *ran-2(t1598)* animals than with wild-type embryos throughout anaphase (Supplemental Figure S5B). We performed OICD only in embryos from homozygous *ran-2(t1598)* mutants for which a metaphase plate eventually formed (Figure 6D). We found a clear increase in the rate of chromatid separation compared with OICD performed in wild-type cells (Figure 6F and Supplemental Figure S5A). Therefore, although a lack of RanGTP reduces chromatid segregation, an excess of RanGTP leads to an increased chromatid separation distance in the absence of centrosomes. Because the distance between chromatids was not affected in *ran-2* mutants with intact centrosomes, we hypothesized that an excess of RanGTP could also influence the length of other spindle microtubules. In this case, the exaggerated force generated by the central spindle would be counterbalanced and masked when the entire spindle remained intact. Overall we have uncovered an important role for RanGTP during *C. elegans* anaphase, which could be detected only after uncoupling the different forces acting on the spindle. Our result show that in addition to its role in spindle formation, RanGTP promotes chromosome segregation during anaphase of mitosis in *C. elegans* embryos, independently of either centrosomes or the traction force exerted by kinetochore microtubules.

DISCUSSION

In *C. elegans* embryos, microtubules can nucleate around DNA, but, in contrast to *Drosophila* or *Xenopus* egg extracts, the autoassembly of a microtubule bipolar spindle requires centrosomes (Bezler and Gönczy, 2010; Toya *et al.*, 2011; and this study, Supplemental Figure S1A). By performing ablation of centrosomes at different time points after NEBD in *C. elegans* embryos, we found that premature destruction of the centrosomes during metaphase prevents chromatid segregation at anaphase. This confirms that microtubules emanating from the centrosomes are needed to capture chromosomes and organize the microtubule spindle in *C. elegans* embryos. Once the spindle is properly formed, however, we found that chromatid segregation could occur in the absence of centrosomes. Therefore, although centrosomes are essential for mitotic spindle formation and spindle positioning, they are dispensable during mitotic anaphase in *C. elegans* embryos. Centrosomes are dispensable during anaphase in many other species and cell types in which centrosomes are either naturally lacking or have been artificially removed (Nicklas, 1989; Khodjakov *et al.*, 2000, 2004; Delattre and Gönczy, 2004; Basto *et al.*, 2006; Zhang and Dawe, 2011). It has been proposed that the pulling activity of kinetochore microtubules is sufficient to drive chromatid segregation in the absence of centrosomes in all these cases. However, kinetochore microtubule activity is unlikely to play a role during anaphase in *C. elegans* embryos (Oegema *et al.*, 2001; Labbe *et al.*, 2004). Our finding that chromatids remain aligned despite the destruction of centrosomes also argues against a mechanism that would allow kinetochore microtubules to pull on individual chromatids independently of one another, as recently described in mammalian cells (Elting *et al.*, 2014; Sikirzhytski *et al.*, 2014). In conclusion, we have shown that chromatid separation during anaphase of *C. elegans* mitosis, like *C. elegans* acentrosomal female meiosis or the segregation of DNA-coated beads on artificial spindles, can proceed independently of the pulling forces exerted by kinetochore microtubules or centrosomes. Our results therefore strongly suggest that an outward pushing force is generated by the spindle midzone to segregate chromatids apart, independently of the presence of centrosomes.

Although this mechanical force leads to a less efficient segregation of chromatids than in cells harboring intact centrosomes, it is sufficient to segregate chromosomes in daughter cells. It was previously observed that chromatids can segregate despite the absence of cortical force generators in *C. elegans* embryos. We propose that the centrosome-independent force is sufficient to segregate chromosomes despite a reduced activity of extrinsic cortical pulling forces. In conclusion, the centrosome-independent mechanical force may constitute a redundant mechanism ensuring the robustness of chromosome segregation in the face of perturbations. Such a force could sustain chromatid segregation in all spindles regardless of their organization (with or without centrosomes, with or without active kinetochore microtubules) and might constitute a default mechanism that has been retained in the course of evolution.

What is the mechanism of action of this centrosome-independent force? We observed that soon after destruction of one or two centrosomes, chromosomes remain properly aligned. This suggests that kinetochore microtubules are still able to hold chromosomes in place despite the absence of centrosomes. We also found that a premature destruction of centrosomes totally abolishes chromosome segregation. Thus the initial bipolar attachment of chromosomes to kinetochore microtubules might be required to allow chromosome movements at later stages in the absence of centrosomes. Therefore, although kinetochore microtubules do not pull on chromatids during anaphase of *C. elegans* embryos, they may serve as a scaffold for other microtubules to generate an outward pushing force. In agreement with this hypothesis, chromosomes cannot segregate in the absence of kinetochores despite the presence of centrosomes in *C. elegans* embryos (Oegema *et al.*, 2001). Moreover, monopolar spindles in *C. elegans* embryos, on which bipolar attachment of chromosomes is not possible, cannot sustain chromatid segregation (Essex *et al.*, 2009). Exploring how an outward force could be generated by midzone microtubules independently of centrosomes and whether this would require the persistence of kinetochore microtubules will be an important challenge for future research. It will also be important to test whether this outward-pushing force is specific to spindles that connect to holocentric chromosomes or is conserved outside nematodes.

Although in this study we did not further explore the mechanical properties of this centrosome-independent force, we identified molecular components that could contribute to its functioning. The plus end–directed molecular motor kinesin-5 is involved in the outward sliding of interpolar microtubules in many different cell types and is therefore a good candidate (Ferenz *et al.*, 2010). However, previous experiments demonstrated that similar to its orthologue in mammalian cells (Collins *et al.*, 2014), the sole *C. elegans* kinesin-5, BMK-1, acts as a brake to oppose the forces generated outside the spindle by cortical pulling forces (Saunders *et al.*, 2007). Using our experimental assay, which allows us to uncouple the mechanical forces acting on the *C. elegans* spindle, we confirmed that BMK-1 prevents chromosome segregation. We showed that BMK-1 acts as a brake to oppose the force generated both inside and outside the central spindle during *C. elegans* anaphase. Furthermore, we confirmed that although the kinesin-6/ZEN-4 accumulates in the spindle midzone to orchestrate cytokinesis, it does not have the ability to slide midzone microtubules outwardly, as previously observed in other cell types (Fu *et al.*, 2009). Of interest, thus far none of the 20 *C. elegans* kinesin-like proteins has emerged as potential candidate for driving spindle elongation. This suggests that their roles in chromosome segregation may be masked by the activity of compensatory forces or redundant proteins. Alternatively, molecular

motors might not be essential to generate an outward-pushing force in the spindle midzone of *C. elegans*.

We found that in the absence of traction forces (due to the destruction of centrosomes), the absence of the major cross-linker of antiparallel microtubules, the MAP-65/SPD-1 protein, led to a faster segregation of chromatids. Thus SPD-1 generates a brake to oppose the force generated by the spindle, independently of the presence of centrosomes. This is in agreement with recent observations made in vitro, demonstrating that the entropic expansion of the yeast MAP-65/Ase1 within the microtubule overlap antagonizes the outward sliding of midzone microtubules (Lansky *et al.*, 2015). By contrast, kinesin-5 and MAP-65 have been shown to act cooperatively to slide midzone microtubules outwardly in the budding yeast (Khmelinskii *et al.*, 2009). An attractive possibility is that the cooperation between these proteins is conserved in *C. elegans*, whereas the orientation of the motor has evolved, leading to an inward displacement of microtubules in wild-type cells.

The microtubule-binding protein CLASP is known to stabilize kinetochore microtubules. We found that inactivation of CLASP totally abolished chromatid segregation in the absence of centrosomes during *C. elegans* mitosis. This result further suggests that kinetochore microtubules might be required to anchor microtubules from the spindle midzone to push on chromatids. However, although CLASP is clearly associated with kinetochores, it is also found enriched in the spindle midzone in different cell types, including the *C. elegans* one-cell embryo (Lemos *et al.*, 2000; Bratman and Chang, 2007; Espiritu *et al.*, 2012). During *C. elegans* female meiosis, CLASP is also required for chromatid segregation in anaphase, whereas kinetochores are dispensable (Dumont *et al.*, 2010). Our results thus raise the interesting possibility that in addition to its role in stabilizing kinetochore microtubules, CLASP may also participate in the outward force by directly stabilizing microtubules of the spindle midzone. We also found that although a reduction of RanGTP decreased chromatid segregation in the absence of centrosomes, an excess of RanGTP led to a striking increase in the rate and extent of chromatid segregation. RanGTP allows the activation of several microtubule-associated proteins and has been shown to organize microtubules and stimulate microtubule growth around DNA in many different organisms (Carazo-Salas *et al.*, 1999; Kalab *et al.*, 1999; Ohba *et al.*, 1999; Wilde and Zheng, 1999). However, due to its role in the early steps of spindle formation, more specific roles of RanGTP during anaphase have rarely been explored (Yokoyama *et al.*, 2009). Our results uncover a clear role for RanGTP during anaphase in *C. elegans* embryos. We propose that RanGTP promotes the activation of microtubule-associated proteins that drive microtubule growth in the central spindle. Overall we uncovered an important function of CLASP and Ran beyond their well-characterized roles in the early steps of spindle formation (Yokoyama *et al.*, 2009; Goshima and Scholey, 2010). The involvement of CLASP and Ran raises the attractive possibility that microtubule growth and polymerization, rather than sliding, might be essential to produce a pushing force at the spindle midzone.

MATERIALS AND METHODS

Strains

Transgenic *C. elegans* fluorescent strains were cultured using standard protocols. The following strains were maintained at 25°C: ANA058 (*oxIs279[pie-1::GFP::histone]* II; *ltIs25[pAZ132, pie-1:GFP::tba-2]*), which was obtained by crossing EG4601 (from the *Caenorhabditis* Genetics Center [CGC]) and OD57 (a kind gift from the Dumont lab), and BN35 (*ran-2(t1598) unc-32(e189)*/qC1 *dpy-19(e1259) glp-1(q339)* III; ltIs37[*pie-1::mCherry::his-58*] IV; ltIs24[*pie-1::GFP::tba-2*]?) was generated by crossing GE2626 (Gönczy *et al.*, 1999) with OD57. *t1598* was identified in a mutational screen for genes affecting cell division processes in the one-cell stage embryo (Gönczy *et al.*, 1999), and we mapped the allele to the *ran-2* gene. Sequencing of the *ran-2* locus in *t1598* animals revealed a G-to-A nucleotide substitution causing a G207R mutation in the RAN-2 protein. We generated ANA065 (*adeIs1*[pMD191, *mex-5::spd-1::GFP*] II) by transposon-mediated homologous recombination (Frøkjaer-Jensen *et al.*, 2008). ANA071 (*adeIs1*[pMD191, *mex-5::spd-1::GFP*] II; ltIs37[pAA64;pie-1::mCherry::HIS-58] IV, *ltIs38*[pAA1;*pie-1::GFP::PH*]) and ANA072 (*adeIs1*[pMD191, *mex-5::spd-1::GFP*] II; ltIs37[pAA64;pie-1::mCherry::HIS-58] IV) were generated by crossing ANA065 to OD95 (from the CGC). DJN001 (*xsEx6[ZEN-4::gfp; rol-6(su1006)]* IV; *?Is?[pie-1::mCherry::tubulin*]) was a kind gift from the Needleman lab. The following strains were maintained at 16°C, and L4 larvae were switched at 25°C for at least 24 h before the experiments: ANA106 (*spd-1(oj5)* I;*oxIs279[pie-1::GFP::histone]* II; *ltIs25[pAZ132, pie-1:GFP::tba-2]*) was generated by crossing WH12 (from the CGC) to ANA058. ANA107 (*zen-4(or153)* IV;*oxIs279[pie-1::GFP::histone]* II; *ltIs25[pAZ132, pie-1:GFP::tba-2]*) was generated by crossing EU554 (from the CGC) to ANA058.

RNAi

RNAi experiments were performed by feeding ANA058 larvae on HT115 bacteria at 25°C (Fraser *et al.*, 2000) for *lin-5 (24h)*, *bmk-1* (30 h), *zen-4* (30 h), *spd-1* (48 h), *cls-2* (44 h), *gpr-1/2* (40 h), and *ran-3* (30 h). *bmk-1*, *zen-4*, *spd-1*, and *cls-2* bacterial clones were from the Ahringer RNAi collection (Kamath *et al.*, 2003). *gpr-1/2* and *lin-5* RNAi clones were a kind gift from the Gönczy lab. *ran-3* RNAi clones were used as in Askjaer *et al.* (2002).

Microscope image acquisition and laser ablation

Embryos were mounted in M9 onto a 2% agarose pad between the slide and the coverslip and recorded on an inverted spinning disk confocal microscope (Leica DMI400; Leica Microsystems, Mannheim, Germany) controlled by MetaMorph (version 7.5.6) and equipped with a 100× immersion objective (HCX PL APO 100×/1.4 oil). Images were acquired with an electron-multiplying charge-coupled device camera (iXion3 897; Andor, Belfast, Northern Ireland) every 0.5–0.78 s, depending on the movie, with an exposure time of 500 ms. For centrosome ablation experiments, a UV laser module (iLas2; Roper, Sarasota, FL) installed on the spinning disk was used ($\lambda = 355$ nm, laser power 45%, number of points, 3500; Onfly module). We also used a spectral confocal microscope (LSM710; Zeiss, Oberkochen, Germany) controlled by the ZEN 2010 software with a 40× oil immersion objective and equipped with an IR two-photon laser (Chameleon; Coherent, Santa Clara, CA; 28% laser power, 50-pixel-diameter ablation zone). Images were acquired with an argon laser, using a 3× numerical zoom. In this study, results obtained after IR ablations are shown only in Supplemental Video S2 and Figure 2A. All other images were obtained after UV irradiation. All panels on the figures, except Supplemental Figure S5, represent an average intensity of three successive frames (*z*-stack projection plug-in of ImageJ [National Institutes of Health, Bethesda, MD]).

Destruction of one centrosome led to the rapid displacement of the remaining spindle toward the opposite side of the cell due to the action of cortical pulling forces acting on the intact centrosome. In contrast, photobleaching of the centrosome did not perturb spindle displacement. Thus rapid centrosome movements constituted a first readout of the disappearance of the centrosome structure. Second, we confirmed the full destruction of the centrosome by observing the next cell division for each targeted embryo. We found

that the cell inheriting the intact centrosome reentered mitosis and formed a bipolar spindle after centrosome duplication, whereas the cell inheriting the ablated centrosome was not able to form a spindle, and no aster re-formed at the second cell cycle (Supplemental Figure S1A). This was expected because neither de novo centriole formation nor autoassembly of a mitotic spindle without centrosomes has been observed in *C. elegans* early embryos (Bezler and Gönczy, 2010; Toya *et al.*, 2011). We also confirmed the destruction of the centrosomes or any microtubule structure that could be in contact with the half-spindle by indirect immunofluorescence (Supplemental Figure S1B). Although some minute asters could be detected in the cell on the same side as the ablated centrosome, they were closely apposed to the cortex and did not contact the rest of the spindle. Finally, we found that the time elapsed between the onset of chromatid segregation and the onset of cytokinesis was statistically similar between ablated and wild-type embryos. Therefore the laser heat did not affect cell cycle progression (Supplemental Figure S1C). Overall the OICD experiments allowed us to fully abolish the effect of the cortical pulling forces by destroying astral microtubules and their contact with the cortex.

For most ablation experiments, the first laser shoot led to the rapid displacement of the remaining spindle (because of cortical pulling forces acting on the intact centrosome). A second shoot was performed 1 or 2 s later if a small aster was still visible on the side of the first ablation to ensure the full destruction of any structure that could contact the "free" chromatid mass. As a consequence, significant photobleaching of fluorescent signals occurred. In all cases, the laser impact generated a plasma in the cytoplasm that was visible in all wavelengths as a bright and compact spot.

Immunofluorescence

Embryos were freeze-cracked as previously described (Colombo *et al.*, 2003) and stained with a mouse monoclonal anti–α-tubulin antibody (1/200; DM1a; Sigma-Aldrich, St. Louis, MO), a rabbit anti–ZYG-9 antibody (1/1000) to mark the centrosomes (a kind gift from the Gönczy lab), and Hoechst (33342; Sigma-Aldrich). Donkey anti-mouse and anti-rabbit secondary antibodies were used at 1/1000 (Jackson ImmunoResearch Laboratories, West Grove, PA). To fix embryos after OICD, embryos were placed on a polylysine-coated coverslip and covered with water. After laser destruction of the centrosome, a coverslip was immediately added onto the embryo, and excess liquid was removed to flatten the embryo. The coverslip sandwich was then immediately frozen on a cold aluminum block and later processed in methanol.

Image analysis and speed calculation

Chromatid and centrosome positions were tracked automatically from the fluorescent movies using a homemade Matlab program. However, because of the photobleaching effects and the plasma generated after laser irradiation, chromatid and centrosome positions were manually tracked using the Manual Tracking plug-in from ImageJ from some movies. Data were later processed using Matlab (Natick, MA).

To extract speed behavior, we first smoothed the chromatid distance curves and calculated the instant speed. From the speed curves, we extracted two discrete speed values. The initial speed corresponds to the maximum speed after the onset of chromatid separation ($t = 0$ s). The final speed corresponds to a second phase, in which the speed is almost constant. This phase starts after the speed of chromatid separation reached its minimal value. For this second phase, we extracted the mean of the speed values (Supplemental Figure S1D).

Statistical tests

Owing to the small sample size of our experiments, we used a Wilcoxon test. A p value of <0.05 was considered statistically significant.

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