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Cyclin E–Cdk2 temporally regulates centrosome assembly and establishment of polarity in *Caenorhabditis elegans* embryos

Carrie R. Cowan[1,2] and Anthony A. Hyman[1]

Establishment of polarity in *C. elegans* embryos is dependent on the centrosome[1]. The sperm contributes a pair of centrioles to the egg and these centrioles remain incapable of polarizing the cortex while the egg completes meiosis. Coincident with the establishment of polarity, the centrioles recruit centrosomal proteins[1], several of which are required for polarity[1–3], suggesting that the temporal regulation of centrosome assembly may control the initiation of polarization. We found that cyclin E–Cdk2 is required for the establishment of polarity. Cyclin E–Cdk2 controls the recruitment of centrosomal proteins specifically at the time of polarity establishment. Cyclin E is required for several examples of asymmetric cell division and fate determination in *C. elegans* and *Drosophila*[4–6]. Here, we suggest a possible mechanism for cyclin E–Cdk2-dependent differentiation: the establishment of cortical polarity by the centrosome.

One-cell *C. elegans* embryos establish an anterior–posterior polarity axis shortly after fertilization (reviewed in refs 7–9). Establishment of this axis involves the creation of two cortical domains, each occupying roughly half the embryo. Before polarization, the entire embryo surface undergoes contractions (ruffling) of a cortical actomyosin meshwork[10]. The actomyosin meshwork redistributes to one half of the embryo[10,11], where it continues to contract, producing ruffles. The other half of the embryo, devoid of the contractile meshwork[10], ceases to ruffle. The domains of contractile polarity correspond with domains of anterior–posterior polarity markers, the partitioning-defective (PAR) proteins (reviewed in refs 8, 12). The PAR-3–PAR-6–PKC-3 complex segregates with the contractile cortex, defining the anterior, and PAR-2 and PAR-1 localize to the smooth cortex, defining the posterior. The centrosome initiates polarity establishment. In embryos in which the centrosome is mechanically or genetically ablated, polarity is not established — ruffling occurs all over the embryo and PAR-2 does not localize to the cortex[1]. The nature of the centrosome signal is unknown.

To identify molecules required for centrosome-induced polarity establishment, we examined genome-wide RNA interference (RNAi)-based differential interference contrast (DIC) microscopy screens[13–16] for lack-of-polarity phenotypes. Contractile polarity can be readily observed in DIC movies and thus served as our initial assay. One gene with an unknown role in polarity establishment was found, cyclin E. Embryos depleted of cyclin E by RNAi (*cye-1(RNAi)* embryos) exhibit ruffling all over the embryo (Fig. 1b) [15]. Homozygous *cye-1* mutants are sterile, producing neither eggs nor sperm [6,17,18], and thus we could not analyze the *cye-1* null phenotype of one-cell embryos. Therefore, the polarity phenotype of *cye-1(RNAi)* embryos was analysed by time-lapse imaging of GFP–PAR-2 and GFP–PAR-6. Consistent with the lack of contractile polarity, in *cye-1(RNAi)* embryos GFP–PAR-2 did not localize to the cortex but remained cytoplasmic during the time of polarity establishment ($n = 16$; Fig. 1b; see Supplementary Information, Movie S1 and Fig. S1). Likewise, in *cye-1(RNAi)* embryos GFP–PAR-6 localized throughout the cortex ($n = 3$; Fig. 1b). Spindle function seemed normal in *cye-1(RNAi)* embryos, although cleavage was delayed relative to wild-type embryos and 50% of *cye-1(RNAi)* embryos divided symmetrically ($n = 16$; see Supplementary Information, Movie S1).

Cyclins regulate the activity of cyclin-dependent kinases (Cdks; reviewed in ref. 19) and cyclin E usually associates with Cdk2. Among the predicted Cdks in the *C. elegans* genome, K03E5.3 shows the highest degree of homology with Cdk2 (ref. 20). *K03E5.3(RNAi)* embryos had a very similar phenotype to *cye-1(RNAi)* embryos — ruffling persisted throughout the cortex, PAR-2 remained cytoplasmic ($n = 6$; Fig. 1c; see Supplementary Information, Movie S1 and Fig. S1), PAR-6 was distributed throughout the cortex ($n = 3$; Fig. 1c) and the first division was sometimes symmetric (33%, $n = 6$; see Supplementary Information, Movie S1). Spindle function seemed normal in K03E5.3-depleted embryos (see Supplementary Information, Movie S1). Thus, we suspect that K03E5.3 is the Cdk regulated by cyclin E during polarity establishment. Hereafter we refer to K03E5.3 as *cdk-2* because of its similarity to Cdk2s from other species.

[1]Max Planck Institute, Molecular Cell Biology & Genetics, Pfotenhauerstrasse 108, 01307 Dresden, Germany.
[2]Correspondence should be addressed to C.R.C. (e-mail: cowan@mpi-cbg.de)

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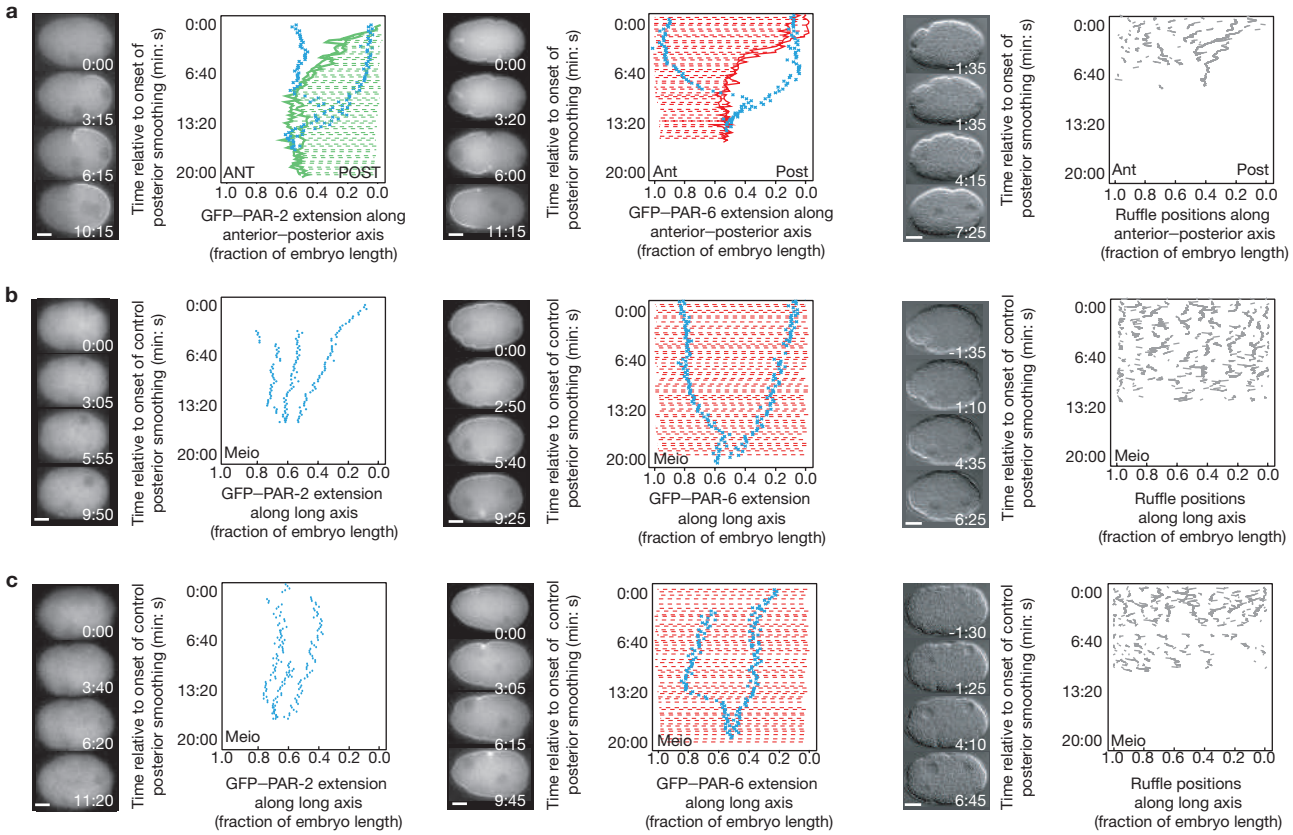

Figure 1 Cyclin E–Cdk2 is required for polarity establishment. (**a**–**c**) Time-lapse images and kymographs of GFP–PAR-2, GFP–PAR-6 and contractile polarity (DIC) in control (**a**), *cye-1(RNAi)* (**b**), and *cdk-2(RNAi)* (**c**) embryos (see also Supplementary Information, Movie S1). Embryo posterior is to the right in **a**; the meiotic pole is to the left in **b** and **c**. The scale bars represent 10 μm. Solid green line, PAR-2 boundaries; dotted green lines, cortical PAR-2; solid red lines, PAR-6 boundaries; dotted red lines, cortical PAR-6; blue x, pronuclei; grey lines, cortical ingressions (ruffles). Anterior (Ant) and posterior (Post) or the meiotic pole (Meio) are indicated on the graphs; all extensions are shown as fractions of the embryo length. In **a**, time is standardized to the onset of posterior smoothing; in **b** and **c**, time 0 was assigned on the basis of a similar cell-cycle stage to that of control embryos as the time of polarization (see Methods).

Currently, lack-of-polarity phenotypes in *C. elegans* embryos can be divided into several general classes (reviewed in ref. 8): first, centrosome defective mutants (for example, *spd-2*, *spd-5* and centrosome ablation) that do not show either PAR polarity or contractile polarity, fail to form spindles and often fail to cleave; second, cortex-defective mutants (for example, *nmy-2*, *mlc-4*, *pfn-1* and *cyk-1*) that do not show PAR polarity, lack all contractile activity, form spindles but fail to cleave; and three, PAR mutants (for example, *par-3*, *par-6*, *pkc-3*) that do not show PAR polarity but do exhibit contractile polarity, form spindles and cleave symmetrically. Cyclin E–Cdk2-depleted embryos do not show PAR polarity or contractile polarity, similar to centrosome-defective mutants; however, they form spindles and divide symmetrically, unlike centrosome-defective mutants but similarly to PAR mutants. *cye-1(RNAi)–cdk-2(RNAi)* mutants show a unique combination of lack-of-polarity phenotypes compared with known polarity establishment mutants, and thus cyclin E–Cdk2 may have a novel role in polarization.

In several organisms and cell lines, cyclin E–Cdk2 has been shown to be involved in regulation of DNA replication, centrosome duplication, S-phase gene expression, histone biosynthesis, pre-mRNA splicing, self-regulation or cell cycle progression[21–23]. These cyclin E–Cdk2-dependent processes proceed through distinct cellular pathways. How do the multiple roles of cyclin E–Cdk2 relate to the role of cyclin E–Cdk2 in polarity establishment? Gene transcription has not been detected in one-cell embryos[24,25] and thus is an unlikely target of cyclin E–Cdk2 in polarity establishment. Cyclin E–Cdk2 depletion in *C. elegans* embryos did not produce a readily detectable defect in centrosome duplication or DNA replication[6] (data not shown). Furthermore, inhibiting centrosome duplication or DNA replication did not affect the establishment of GFP–PAR-2 polarity (see Supplementary Information, Fig. S2). However, cyclin E–Cdk2 depletion caused a slight deviation from the normal rate of cell-cycle progression (see Supplementary Information, Movie S1 and Fig. S2).

Could the cell-cycle delay be responsible for the lack of polarity establishment in cyclin E–Cdk2 depleted embryos? The consequence of cell-cycle delay for the establishment of polarity was determined by depleting the other essential cyclins in *C. elegans* ($n \geq 3$ for each gene; Fig. 2and see Supplementary Information, Movie S2). *cyb-2.2(RNAi)*, *cyb-3(RNAi)* and *cyh-1(RNAi)* embryos showed cell-cycle delays but established PAR-2 polarity (Fig. 2 and see Supplementary Information, Movie S2 and Fig. S2)[26]. Additionally, A-type cyclin *ZK507.6(RNAi)* and *cyb–2.2(RNAi)* embryos showed meiotic defects as evident from the presence of extra pronuclei (similarly to *cye-1(RNAi)–cdk-2(RNAi)*embryos), but established PAR-2 polarity (Fig. 2 and see Supplementary Information, Movie S2). Neither the cell-cycle delay nor the meiotic defect in cyclin

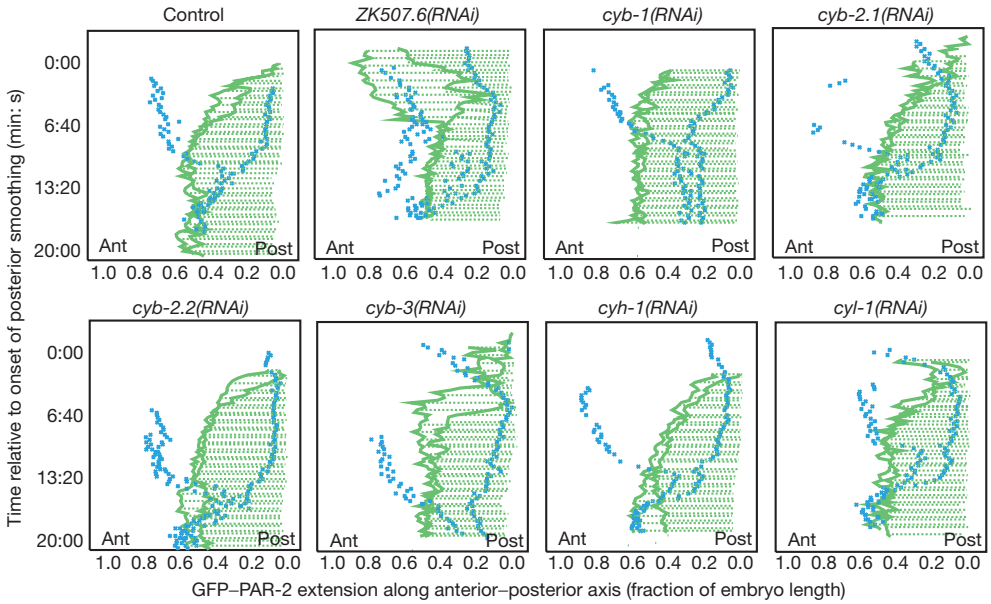

Figure 2 Cell-cycle delays associated with depletion of other cyclins do not prevent polarity establishment. Kymographs of GFP–PAR-2 polarity in control, a predicted A-type cyclin *ZK507.6(RNAi)*, B-type cyclin *cyb-1(RNAi)*, B-type cyclin *cyb-2.1(RNAi)*, B-type cyclin *cyb-2.2(RNAi)*, B-type cyclin *cyb-3(RNAi)*, cyclin H *cyh-1(RNAi)* and cyclin L *cyl-1(RNAi)* embryos (see Supplementary Information, Movie S2). Solid green lines, PAR2 boundaries; dotted green lines, cortical PAR-2; blue x, pronuclei. Because the centroids of the pronuclei were tracked, the male and female pronuclei remain separated by one nuclear diameter (~0.15 of embryo length) at pronuclear meeting (~10:30 in the control embryo shown). Anterior (Ant) and posterior (Post) are indicated on the graphs; all extensions are shown as fractions of the embryo length. Time is standardized to the onset of posterior smoothing. The large polar body in the *cyb-3(RNAi)* embryo (see Supplementary Information, Movie S2) is not included in the kymograph. Delays in cell-cycle timing following depletion of *cyb-2.2*, *cyb-3* and *cyh-1* are apparent in the delayed pronuclear meeting. Meiotic defects are evidenced by an additional pronuclear trace *ZK507.6(RNAi)*, *cyb-2.2(RNAi)*). *ZK507.6(RNAi)* embryos failed to eliminate PAR-2 from the cortex during meiosis such that the cortical PAR-2 domain shrank rather than expanded during polarity establishment. The timing of polarization was normal.

E–Cdk2-depleted embryos is likely to prevent embryo polarization. These data suggest a novel function for cyclin E–Cdk2 during the establishment of polarity.

The initial lack-of-polarity phenotype observed in cyclin E–Cdk2-depleted embryos seemed similar to the centrosome defective mutants *spd-2* and *spd-5* (refs 1–3). We wanted to determine whether cyclin E–Cdk2 is required for the recruitment of SPD-2 or SPD-5 to the centrosome. Embryos were depleted of *cyclin E* or *Cdk2* using RNAi and the amount of centrosomal SPD-2 and SPD-5 was quantified by immunofluorescence microscopy (Fig. 3 and see Supplementary Information, Fig. S3). PAR-2 localization was used to judge whether polarity had been established and chromatin was stained to estimate cell-cycle time by comparison with histone–GFP movies (see Methods). In meiotic *cye-1(RNAi)–cdk-2(RNAi)* embryos, centrosomal SPD-2 and SPD-5 levels were normal. In early and late prophase *cye-1(RNAi)–cdk-2(RNAi)* embryos, the centrosomal SPD-5 and SPD-2 levels were reduced and SPD-5 was more severely affected than SPD-2. In mitotic *cye-1(RNAi)–cdk-2(RNAi)* embryos, centrosomal SPD-2 and SPD-5 levels were again normal. Thus, cyclin E–Cdk2 seems to be required for recruitment of SPD-5 and SPD-2 to the centrosome during prophase. As both SPD-5 and SPD-2 are required for polarity establishment, cyclin E–Cdk2-mediated centrosomal protein recruitment may directly control polarity establishment, although the possibility of centrosome-independent roles for cyclin E–Cdk2 during polarization cannot be excluded. However, *spd-5(RNAi)*, *spd-2(RNAi)*, *cye-1(RNAi)* and *cdk-2(RNAi)* embryos show indistinguishable phenotypes with respect to polarity establishment[1] (see Supplementary Information, Fig. S1), and double mutant combinations show the same lack of cortical PAR-2 localization (see Supplementary Information, Fig. S1), further supporting the belief that the lack of polarity in *cye-1(RNAi)–cdk-2(RNAi)* embryos most likely results from the failure to recruit centrosomal proteins.

Two possibilities may explain the apparent prophase-specificity of the centrosome defect: centrosomal protein accumulation is slow in *cye-1(RNAi)–cdk-2(RNAi)* embryos, or centrosomal protein accumulation is late in *cye-1(RNAi)–cdk-2(RNAi)* embryos. Slow centrosomal protein accumulation could suggest that cyclin E–Cdk2 is a general centrosome assembly factor, likely required throughout the cell cycle, whereas delayed centrosomal protein accumulation could suggest that cyclin E–Cdk2 is required during a specific time in the cell cycle — for example during the establishment of polarity. To distinguish these possibilities, the kinetics of centrosomal protein recruitment in cyclin E–Cdk2 depleted embryos were analysed by time-lapse imaging and quantification of GFP-tagged centrosomal protein recruitment. In wild-type embryos, the amounts of centrosomal SPD-2, γ-tubulin and the Aurora kinase AIR-1 increased steadily throughout the mitotic cell cycle (Fig. 3b). However, in *cye-1(RNAi)–cdk-2(RNAi)* embryos, the amounts of centrosomal SPD-2, γ-tubulin and AIR-1 did not increase from the level observed on entry into the mitotic cell cycle until approximately ten minutes after control-embryo polarization, after which centrosomal proteins reached approximately wild-type levels (Fig. 3c, see Supplementary Information, Fig. S1, and data not shown). In *spd-2(RNAi)* and *spd-5(RNAi)* embryos, the amount of centrosomal protein never increased beyond the level observed on entry into the mitotic cell cycle (see Supplementary Information, Fig. S3). Although we cannot exclude that residual cyclin E–Cdk2 activity may contribute to centrosome recovery, our data suggests that cyclin E–Cdk2 has a dominant role in

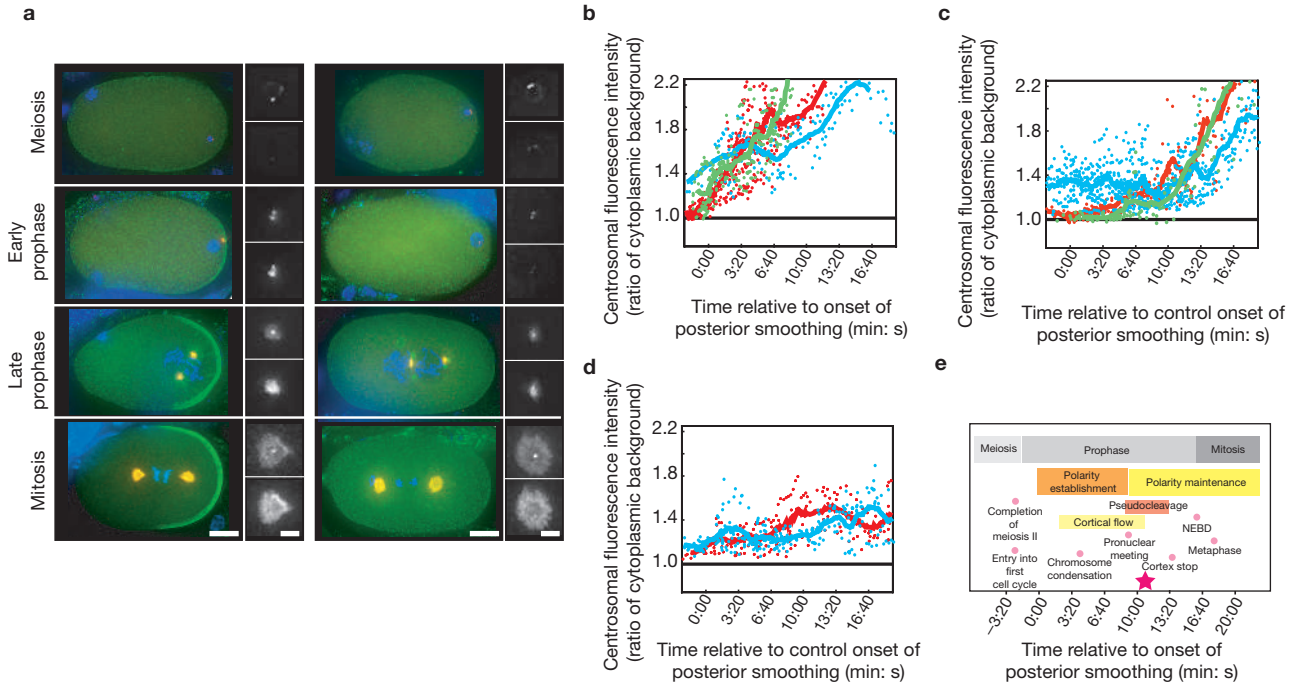

Figure 3 Cyclin E–Cdk2 is required for centrosomal protein recruitment during polarity establishment. (**a**) Embryos were sorted into four temporal stages (see Methods) representing meiosis, early prophase, late prophase and mitosis. SPD-2, red; SPD-5, yellow; PAR-2, green; DNA, blue. Images are projections of deconvolved *z*-sections encompassing the middle half of the cell. In control embryos, posterior is to the right. In *cye-1(RNAi)–cdk-2(RNAi)* embryos, the meiotic pole is to the left. The scale bars represent 10 μm. Magnifications correspond to the centrosomal region of SPD-2 (top) and SPD-5 (bottom); single deconvolved *z*-sections are displayed. The scale bars represent 2.5 μm in the magnifications. During meiosis, SPD-2 is predominantly localized on the centrosomes (bright dots) but also detectable in a ring around the condensed sperm chromatin. Cyclin E–Cdk2 depleted embryos show premature separation of centrioles. (**b**–**d**) Recruitment of the centrosomal proteins SPD-2 (blue), AIR-1 (green), and γ-tubulin (red) in control (**b**), *cye-1(RNAi)* (**c**), and *air-1(RNAi)* (**d**) embryos. In **a**, time is standardized to the onset of posterior smoothing; in (**b**–**d**), time 0 was assigned on the basis of a similar cell-cycle stage to that of control embryos as the time of polarization (see Methods). (**e**) The timing of events pertaining to the mitotic cycle and cortical polarity during the first cell cycle (see Methods). Centrosome recovery in *cye-1(RNAi)–cdk-2(RNAi)* embryos occurred approximately ten minutes after polarity establishment in control embryos, indicated with a pink star.

centrosome assembly during polarity establishment, and that the molecular requirements for centrosome assembly change during the cell cycle. AIR-1 seems to be a general centrosome-assembly factor and is essential for centrosome maturation during mitosis[27]. In *air-1(RNAi)* embryos the amount of centrosomal proteins accumulated gradually throughout the cell cycle, but at much lower levels than in wild-type embryos (Fig. 3d). Depletion of cyclin E or Cdk2 together with AIR-1 leads to centrosomes with reduced levels of SPD-2 relative to either *cye-1(RNAi)–cdk-2(RNAi)* or *air-1(RNAi)* embryos alone (see Supplementary Information, Fig. S3). Thus, cyclin E–Cdk2 is required for centrosome assembly at the time of polarity establishment, but a different mechanism, mediated in part by Aurora kinase, is responsible for centrosome assembly thereafter.

It is unclear whether the transition between cyclin E–Cdk2-dependent and cyclin E–Cdk2-independent centrosomal protein recruitment corresponds to any known event in the first cell cycle. We correlated the times at which various events occur in wild-type one-cell embryos with centrosome recovery in *cye-1(RNAi)–cdk-2(RNAi)* embryos (Fig. 3e). Of the events measured, three correlated with centrosome rescue: the onset of the polarity maintenance phase, pronuclear meeting and pseudocleavage. This time point may represent a cell-cycle-defined transition in how contractility and centrosome assembly are regulated. The correlation between the time of centrosome recovery in *cye-1(RNAi)–cdk-2(RNAi)* embryos and the transition to the polarity-maintenance phase may explain the observation that many *cye-1(RNAi)–cdk-2(RNAi)* embryos did not establish polarity, even though centrosome assembly recovers (Fig. 1, and see Supplementary Information, Fig. S1). Changes in the cortical actomyosin organization that occur at the transition to polarity maintenance[10] may not support the normal polarity establishment mechanism by the centrosome.

Consistent with the temporally defined requirement for cyclin E–Cdk2 for centrosome assembly, cyclin E–Cdk2 seemed to partly localize with centrosomes before polarity initiation. Immunofluorescent localization of CYE-1, YFP–CDK-2 and SAS-4 (a centriole marker) indicated that CYE-1 and CDK-2 colocalize around the sperm chromatin in a small (<2 μm) ring with an intense focus at the centrioles (Fig. 4a, $n = 18$). Depletion of either cyclin E or CDK-2 prevented the localization of cyclin E–Cdk2 to the sperm chromatin–centriole ring before the polarity establishment phase (Fig. 4; *cye-1(RNAi)*, $n = 7$; *cdk-2(RNAi)*, $n = 7$). Depletion of either SPD-5 or SPD-2 did not affect the localization of cyclin E–Cdk2 to the sperm chromatin–centriole ring (Fig. 4b, c; *spd-5(RNAi)* GFP–CYE-1, $n = 6$; YFP–CDK-2, $n = 5$; *spd-2(RNAi)* GFP–CYE-1, $n = 6$; YFP–CDK-2, $n = 6$), suggesting that cyclin E–Cdk2 functions upstream of SPD-5 and SPD-2 at the centrosome. On entry into the mitotic cell cycle, neither CYE-1 nor CDK-2 remained associated with the centrosomes; instead, both proteins localized to the male and female pronuclei (Fig. 4b, c; GFP–CYE-1, $n = 6$; YFP–CDK-2, $n = 6$). Although several caveats exist (for instance centrosomal cyclin E–Cdk2 may be undetectable later in the cell cycle and centrosomal

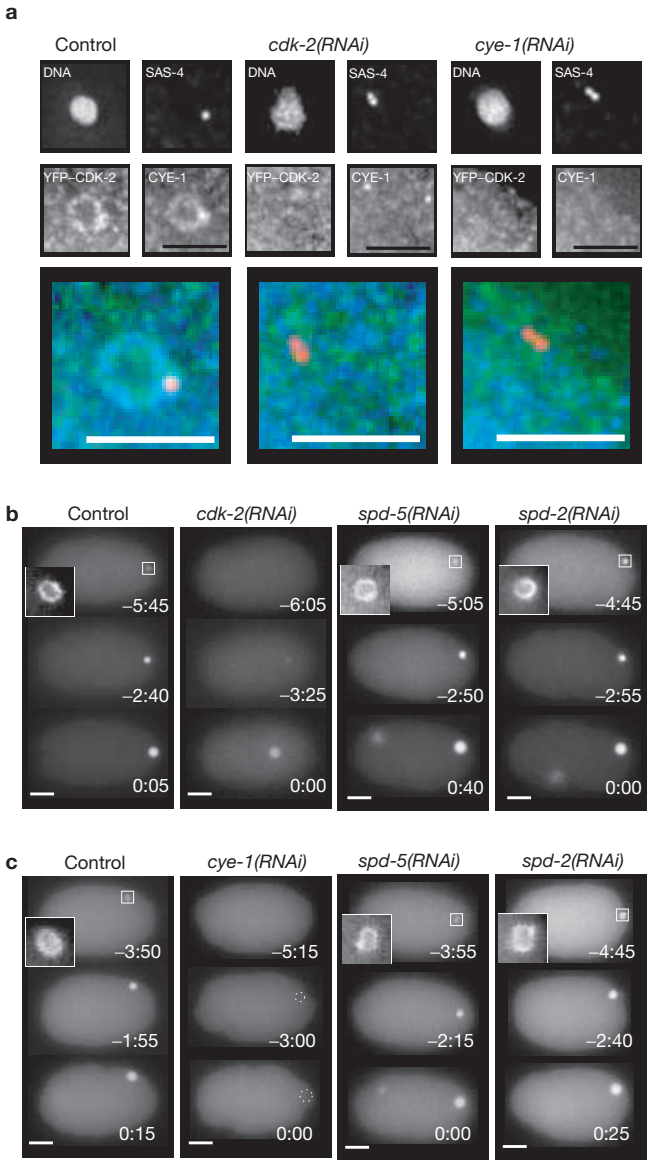

Figure 4 Cyclin E and Cdk2 are mutually required for colocalization around the sperm chromatin/centrioles before polarity establishment, independently of SPD-5 and SPD-2. (**a**) Immunofluorescent localization of CYE-1 (green), YFP-CDK-2 (blue) and the centriole protein SAS-4 (red). Images are projections of deconvolved *z*-sections encompassing ~1 μm. The scale bars represent 2.5 μm. Image intensity was scaled to show cytoplasmic staining, which seems to be non-specific background (data not shown). The sperm chromatin–centrosome ring of CYE-1 and CDK-2 is similar to SPD-2 localization during meiosis. (**b**, **c**) Time-lapse images of GFP-CYE-1 (**b**) and YFP-CDK-2 (**c**) in control, *cdk-2(RNAi)*, *cye-1(RNAi)*, *spd-5(RNAi)*, and *spd-2(RNAi)* embryos. Embryo posterior is to the right (control); the meiotic pole is to the left (*cye-1(RNAi)*, *cdk-2(RNAi)*; *spd-5(RNAi)*; *spd-2(RNAi)*). Scale bars, 10 μm. In controls, time is standardized to the onset of posterior smoothing; in *cye-1(RNAi)*, *cdk-2(RNAi)*, *spd-5(RNAi)* and *spd-2(RNAi)*, time 0 was assigned on the basis of a similar cell cycle stage to that of control embryos as the time of polarization (see Methods). In **c**, the position of the male pronucleus in *cye-1(RNAi)* embryos, judged from the corresponding DIC movies, is indicated by a circle.

cyclin E–Cdk2 may not be active), the apparent absence of cyclin E–Cdk2 from centrosomes after polarity initiation is consistent with our finding that cyclin E–Cdk2 regulates a phase of centrosome assembly required for polarity establishment. The temporally defined requirement for cyclin E–Cdk2 activity and its transitory centrosomal localization are consistent with a model in which centrosomal cyclin E–Cdk2 initiates centrosome assembly but is not involved in the assembly process itself.

Cyclin E functions in generating asymmetry in several other developmental contexts. In *C. elegans*, cyclin E influences cell-fate decisions in vulval precursor cells, possibly through an effect on cell-cycle length[6]. In the differentiation of glial cells in *Drosophila*, cyclin E is likewise required for correct fate determination, but independently of cell-cycle control[5]. Similarly, gonad development in *C. elegans* requires cyclin D activity for cell-fate determination following asymmetric division, but independent of cell-cycle regulation[28]. Here, we also show a cell cycle-independent role for cyclin E–Cdk2 in asymmetric cell division. Cyclins may be conserved regulators of asymmetric differentiation, perhaps independently of general cell-cycle control.

It is not clear how cyclin E controls cell-fate decisions. We have shown that cyclin E–Cdk2 is required for centrosome assembly and polarity establishment in *C. elegans*. The lack of polarity in *cye-1(RNAi)–cdk-2(RNAi)* embryos leads to a failure in fate determination: anterior and posterior identities are not correctly defined and thus, fate determinants are not segregated (see Supplementary Information, Fig. S2). Whether the fate-determination mechanism of cyclin E–Cdk2 in the *Drosophila* nervous system[5] or *C. elegans* vulva cells[6] relies on the establishment of cortical polarity, as in *C. elegans* one-cell embryos, remains to be determined. A role for cyclin E–Cdk2 in asymmetric cell divisions or fate determination in vertebrates has yet to be uncovered. In vertebrates, centrosome duplication requires cyclin E–Cdk2 (refs 29, 30), for which cyclin E–Cdk2 could conceivably function through the recruitment of centrosomal proteins necessary to trigger duplication. The phenotype of cyclin E–Cdk2-depleted *C. elegans* embryos may therefore provide a link between the role of cyclin E in fate determination and centrosome assembly: in *C. elegans* embryos, fate determination is largely controlled by cortical polarity, and the establishment of cortical polarity requires centrosomal protein recruitment.

Cyclin E–Cdk2 has been implicated in a wide range of cellular processes involving several cellular pathways. One possibility is that cyclin E–Cdk2 directly regulates numerous targets. A second possibility is that the spectrum of cyclin E–Cdk2-dependent processes is controlled by a single 'master' pathway — centrosome assembly may be the master pathway by which cyclin E controls downstream processes. The role of cyclin E in progression through the G1–S restriction point has been shown to require centrosomes[31], and specifically the recruitment of centrosomal proteins[32]. However, links between centrosomes and DNA replication or pre-mRNA splicing have not been investigated. Cyclin E–Cdk2 can regulate the DNA replication machinery directly[21], suggesting more complexity than a single master pathway would entail. The most generalized function of cyclin E–Cdk2 seems to be the ability to respond to cyclin A/B–Cdk1 activity levels and thereby provide temporal control for a variety of cellular machines. □

METHODS

Worm strains, time-lapse imaging and image display. All worm strains were maintained at 16 °C and shifted to 25 °C for 18–26 hours before recording (seee Supplementary Information ,Table S1). Embryo recording was performed at 19–22 °C. Embryos were dissected and mounted using 0.1 M NaCl, 4% sucrose ± 2% agarose. Wide-field GFP and DIC movies were recorded at 10, 15 or 25 s intervals (with no apparent differences) using either a 40× 1.3NA or 60× 1.4NA objective lens, as previously described[1], except for the ruffle tracking experiments (Fig. 1)

in which DIC movies were recorded at 5 s intervals. The following strains were used: N2 (wild type), AZ212 (GFP–histone H2B), DH1 [*zyg-1(b1ts)*], JH1380 (GFP–PAR-2), TH25 (GFP–PAR-6), TH27 (GFP–γ-tubulin), TH42 (GFP–SPD-2), TH49 (GFP–PAR-2; GFP–SPD-2), TH41 (GFP–AIR-1), TH59 (GFP–CYE-1) and TH98 (YFP–CDK-2). Aminoterminal GFP-tagged AIR-1, GFP-tagged CYE-1 and YFP-tagged CDK-2 are full-length genomic clones (WormBase release WS150; http://ws150.wormbase.org) expressed under the *pie-1* promoter in the pAZ132 vector (gift from A. Pozniakowski, Max Planck Institute, Molecular Cell Biology and Genetics (MPI-CBG), Dresden, Gemany). Transgenic worms were created by microparticle bombardment. Images were processed as 8-bit tiffs using GraphicConverter X v5.4 (www.lemkesoft.com); linear scaling was applied.

Data quantification and analysis. Data quantification and analysis was performed with GNU Octave (2.1.53; http://www.octave.org) and Metamorph/Metavue (Molecular Devices, Downingtown, PA). PAR-2 and ruffle kymographs were generated as described previously[1]. Means ± s.d. are given; sample sizes are given in the text. The data was analysed for sameness using a two-tailed Student's *t*-test.

In embryos in which polarity was established, time 0 was assigned based on the first evidence of progressive cortical smoothing (cessation of ruffling), the earliest indicator of polarity establishment. Time 0 occurred roughly 2:30 (min: s) after completion of meiosis in control embryos, at which time the radius of the male pronucleus was ~2 μm. The size of the male pronucleus, as a function of time elapsed from completion of meiosis, was highly reproducible from embryo to embryo, both in control and various RNAi embryos (see Supplementary Information, Fig. S2). Additionally, the rate of male pronucleus growth was largely unchanged in embryos in which the cell-cycle timing was significantly delayed, such as *cye-1(RNAi)* and *cyb-3(RNAi)* embryos (see Supplementary Information, Fig. S2). Therefore, in embryos in which polarity was not established, time 0 was assigned based on the size of the male pronucleus (~2 μm radius) and thus, indirectly, the time elapsed from completion of meiosis.

To calculate the percent of embryos with cortical GFP–PAR-2, one-cell embryos were classified as 'polarity establishment' or 'polarity maintenance' (see Fig. 3e) based on pronuclear size and/or appearance, as estimated by comparing the timing of cell-cycle events (Fig. 3e) with the radial increase of the male pronucleus (see Supplementary Information, Fig. S2). Cortical PAR-2 localization was considered positive if there was a local cortical region of higher fluorescence intensity than the surrounding regions and if this region occupied more than approximately 5% of the cortex.

Cell-cycle timing in control, *cye-1(RNAi)*, *cdk-2(RNAi)* and *cyb-3(RNAi)* embryos was determined from GFP–histone H2B and the corresponding DIC time-lapse images. The end of meiosis II was defined as the start of decondensation of the maternal chromatin following meiotic division. Polarity establishment corresponds to the onset of posterior cortical smoothing or a similar stage based on the size of the male pronucleus in genotypes in which polarity was not established (see above). The onset of chromosome condensation in the male pronucleus was defined by evidence of non-uniform GFP–histone H2B fluorescence (the first appearance of 'threads'). Cortex Stop was assigned when cortical movements were no longer evident in DIC images. Cortex Stop is significantly later than the end of ruffle kymographs in Fig. 1, as the kymograph plotting method filters out small transient ruffles that account for the later ruffling or cortical movement events. Nucler envelope breakdown (NEBD) was calculated based on a decrease in the intensity of diffuse (non-chromosomal) nuclear GFP–histone H2B.

Centrosomal accumulation of GFP-tagged proteins was analysed as previously described[1] and lines represent a sliding average (window size 20) of the data points (dots). Briefly, time-lapse images of GFP-tagged centrosomal proteins were used to quantify fluorescence intensity in one-cell embryos. Centrosomal fluorescence is represented as the ratio of cytoplasmic background (1.0 represents no signal). The data represent a minimum of five embryos per centrosome marker and RNAi treatment.

The times of various events in one-cell embryos were calculated from time-lapse imaging of GFP–histone, GFP–PAR-2 or GFP–PAR-6 in control and *par-2(RNAi)* embryos (polarity maintenance), and from the literature[33]. The transition from meiosis to mitotic prophase was defined by decondensation of the maternal chromatin following the second meiotic division. The transition from prophase to mitosis was defined by the time of NEBD (see earlier descriptions). Polarity establishment refers to the stage during which the posterior domain is expanding and cortical PAR-2 levels increase. Polarity maintenance refers to the time after which PAR-2 activity becomes essential for restricting the anterior PAR complex to the anterior cortex[33], which approximately coincides with meeting of the male and female pronuclei.

RNA interference. The primer information and method of RNAi are given in the Supplementary Information, Table S1. Most experiments were performed using both *cye-1(RNAi)* embryos and *cdk-2(RNAi)* embryos and gave similar results. In some cases, data was grouped or only one phenotype was shown for simplicity, referred to as *cye-1(RNAi)–cdk-2(RNAi)* or cyclin E–Cdk2 depleted. The centrosomal recruitment kinetics of SPD-2, AIR-1 and γ-tubulin were analysed in *cye-1(RNAi)* embryos, shown in Fig. 3c. SPD-2 and γ-tubulin recruitment were analysed in *cdk-2(RNAi)* embryos and showed a similar defect to *cye-1(RNAi)* (data not shown). SPD-2 recruitment in *cdk-2(RNAi)* embryos is shown in the Supplementary Information, Figs S1 and S3.

The predicted cyclins in the *C. elegans* genome were determined from WormBase (release WS140; http://ws140.wormbase.org) using the INTERPRO motif 'cyclin' (IPR006670). Cyclins were considered essential if RNAi and/or mutant phenotypes included embryonic lethality, as annotated in WormBase.

Immunofluorescence microscopy and quantification. Immunofluorescence microscopy was performed as described using PAR2a (1:100), SAS-4 (1:500), CYE-1 (1:1000, gift from E. Kipreos, University of Georgia, Athens, GA), GFP (1:1000, gift from L. Pelletier, MPI-CBG), P granule (1:500, gift from S. Strome, University of Indiana, Bloomington, IN) antibodies, visualized using fluorescent-conjugated secondary antibodies (Jackson Immunochemicals, Newmarket, UK), and directly labelled SPD-2 and SPD-5 antibodies, for intensity quantifications. Wide-field images were acquired and three-dimensionally deconvolved as previously described[1]. Only embryos with no restricted PAR-2 localization were analysed for P granule localization. For centrosomal protein quantification, embryos were classified into four temporal groups based on nuclear size, nuclear/centrosome position and chromatin appearance, determined as described in the Supplementary Information, Fig. S1(meiosis, embryos with condensed maternal chromatin undergoing meiotic divisions; early prophase, embryos in which the male chromatin had decondensed (relative to meiosis) but such that both male and female chromosomes showed no or little condensation — the male and female pronuclei were at a distance from each other and the two centrosomes associated with the male pronucleus were not fully separated; late prophase, embryos in which pronuclei were adjacent to each other and chromosome condensation had progressed so that distinct threads were visible — centrosomes were associated with the apposed pronuclei; mitosis, embryos in which chromosomes had aligned between or segregated towards the centrosomes).

For quantification of the levels of centrosome SPD-2 and SPD-5, a single *z*-section from undeconvolved image *z*-stacks representing the brightest centrosomal intensity of SPD-2 was chosen for analysis of the centrosome (each centrosome was treated separately). The entire centrosome signal (either SPD-2 or SPD-5) was circled and the integrated intensity calculated. The same size circle in a cytoplasmic region was used to standardize integrated intensity values. To calculate the ratio of SPD-2 to SPD-5, the average intensity of the centrosomal signal (integrated intensity per pixel area) was determined; the SPD-2 value was divided by the SPD-5 value for independent centrosomes. The data was analysed for statistical significance using a two-tailed Student's *t*-test. *P* values less than 0.05 were considered significant.

Note: Supplementary Information is available on the Nature Cell Biology website.

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COMPETING FINANCIAL INTERESTS

The authors declare that they have no competing financial interests.

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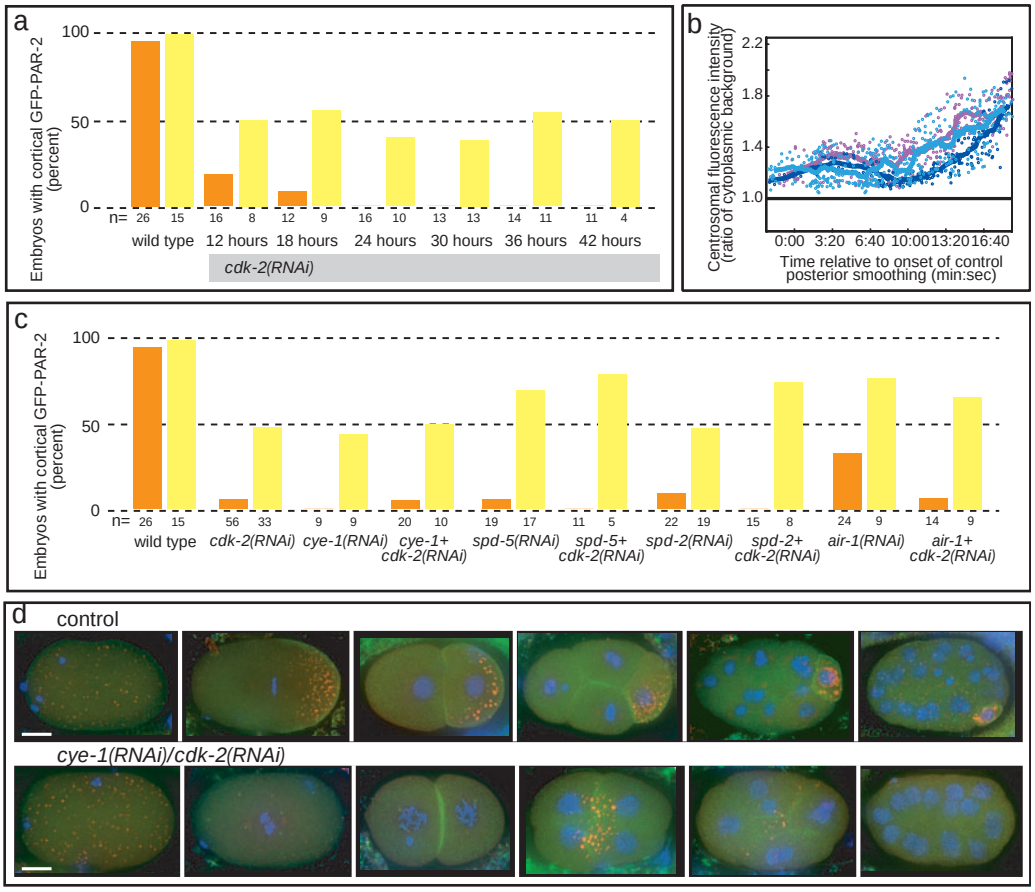

Figure S1 Cyclin E-Cdk2 is required to establish polarity. **(a and c)** Percent of embryos with cortical PAR-2 localization during polarity establishment (orange) and polarity maintenance (yellow) phases (see Materials and Methods and Fig. 3e). The 4% of wild type "polarity establishment" embryos that was not PAR-2 positive reflects one early embryo that showed low GFP-PAR-2 intensity. **(a)** Cortical PAR-2 localization is not affected by an increased *cdk-2(RNAi)* incubation period. *cdk-2(RNAi)* incubation periods greater than 42 hours produced pronounced sterility, and thus we could not assess a more severe *cdk-2* loss-of-function. **(b)** The time of centrosome recovery in cyclin E-Cdk2 depleted embryos is not affected by an increased *cdk-2(RNAi)* incubation period. Centrosomal SPD-2 after 12 hours (purple), 24 hours (light blue), and 36 hours (dark blue) *cdk-2(RNAi)*. Centrosome recovery begins around ten minutes in embryos from all three *cdk-2(RNAi)* incubation times. Centrosome recovery in *cdk-2(RNAi)* and *cye-1(RNAi)* embryos occurs at the same cell cycle time (ten minutes after control polarity establishment; Fig. 3c) although SPD-2 recruitment in *cdk-2(RNAi)* embryos occurs slightly slower than in *cye-1(RNAi)* embryos. **(c)** Cyclin E-Cdk2 depletion prevents PAR-2 polarity establishment similarly to depletion of the centrosomal proteins SPD-5 and SPD-2. The late PAR-2 localization observed in *spd-5(RNAi)* and *spd-2(RNAi)* embryos is consistent with previous data on *spd-5(or213ts)* and *spd-2(oj29)* mutants[1,2]. **(d)** Localization of P granule, germ line fate determinants, in control and *cye-1(RNAi)/cdk-2(RNAi)* embryos. P granules, red; PAR-2, green; DNA, blue. Images are projections of deconvolved z-sections encompassing roughly three quarters of the cellular volume. In control embryos, posterior is to the right; in *cye-1(RNAi)/cdk-2(RNAi)* embryos, the meiotic pole is to the left. Scale bars, 10 μm. PAR-2 localizes to the P lineage (germ line precursors) and to cell-cell contacts of committed blastomeres. In wild type embryos, P granules were restricted to the posterior of post-meiotic one-cell embryos and to the P lineage cells thereafter (n=16). In *cye-1(RNAi)/cdk-2(RNAi)* embryos, P granules were either undetectable (50%) or distributed uniformly in one-cell embryos or to all cells in multi-cellular embryos (50%; n=24). The cortical PAR-2 localization in some *cye-1(RNAi)/cdk-2(RNAi)* embryos may represent cell-cell contacts and appeared able to recruit P granules.

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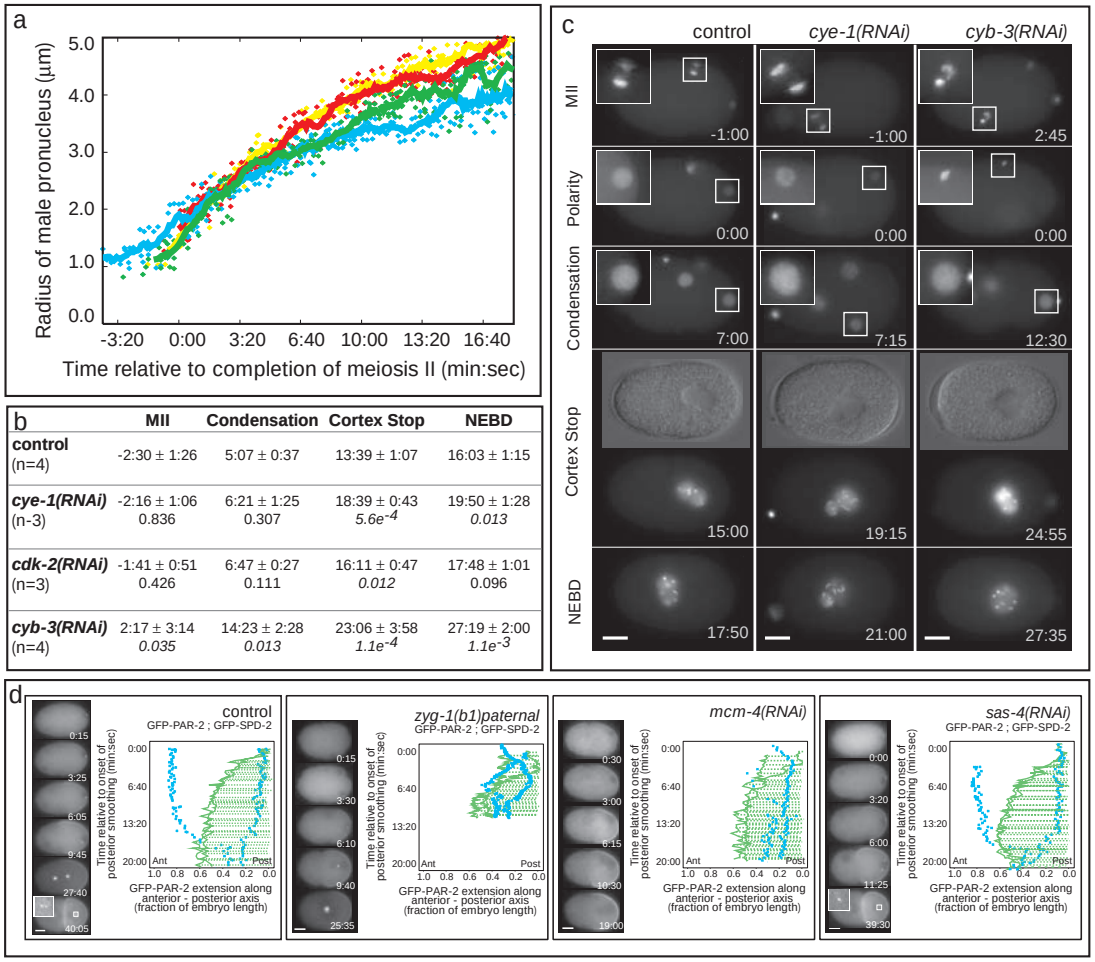

b

	MII	Condensation	Cortex Stop	NEBD
control (n=4)	-2:30 ± 1:26	5:07 ± 0:37	13:39 ± 1:07	16:03 ± 1:15
cye-1(RNAi) (n-3)	-2:16 ± 1:06 0.836	6:21 ± 1:25 0.307	18:39 ± 0:43 *5.6e^{-4}*	19:50 ± 1:28 *0.013*
cdk-2(RNAi) (n=3)	-1:41 ± 0:51 0.426	6:47 ± 0:27 0.111	16:11 ± 0:47 *0.012*	17:48 ± 1:01 0.096
cyb-3(RNAi) (n=4)	2:17 ± 3:14 *0.035*	14:23 ± 2:28 *0.013*	23:06 ± 3:58 *1.1e^{-4}*	27:19 ± 2:00 *1.1e^{-3}*

Figure S2 (a) Change in pronuclear size as a cell-cycle independent timer. The radius of the male pronucleus was calculated during the first embryonic cell cycle following the completion of meiosis II, as judged by the decondensation of maternal chromosomes using GFP-histone time-lapse recordings (control, n=4; *cye-1(RNAi)*, n=3; *cdk-2(RNAi)*, n=3; *cyb-3(RNAi)*, n=4). Control, green; *cye-1(RNAi)*, red; *cdk-2(RNAi)*, yellow; *cyb-3(RNAi)*, blue. Nuclear radius increases regardless of alterations in the cell cycle timing of other events in *cye-1(RNAi)*, *cdk-2(RNAi)*, and *cyb-3(RNAi)* embryos. Dots represent individual measurements; lines are running averages of the data set (window size: 0.1 of data points). (b-c) Defects in general cell cycle timing do not affect polarity establishment. MII: completion of meiosis II. Condensation: onset of chromosome condensation in the male pronucleus. Cortex Stop: cessation of cortical activity preceding mitosis. NEBD: nuclear envelope breakdown. **(b)** Time intervals were calculated relative to polarity establishment (control and *cyb-3(RNAi)*) or to a similar developmental time (*cye-1(RNAi)* and *cdk-2(RNAi)*; Materials and Methods). Data were tested for difference from wild type (P values are listed below the times); significant differences are indicated in italics. *cdk-2(RNAi)* embryos have a less marked cell cycle delay that *cye-1(RNAi)* embryos, perhaps suggesting a Cdk2-independent role for cyclin E in cell cycle regulation, as exists in mammalian cells [1]. **(c)** Time-lapse images of GFP-histone H2B in control, *cye-1(RNAi)*, and *cyb-3(RNAi)* embryos. Embryo posterior is to the right in control and *cyb-3(RNAi)* embryos; the meiotic pole is to the left in *cye-1(RNAi)* embryos. Time is standardized to the onset of posterior smoothing in control and *cyb-3(RNAi)* embryos and to a similar cell cycle stage in *cye-1(RNAi)* embryos (Materials and Methods). Scale bars, 10 µm. (d) Defects in centrosome duplication and DNA replication do not affect polarity establishment. Time-lapse images and kymographs of GFP–PAR-2 polarity in control, *zyg-1(b1ts)paternal*, *sas-4(RNAi)*, and *mcm-4(RNAi)* embryos. In *zyg-1(b1ts)paternal*, and *sas-4(RNAi)* experiments, the centrosomal marker GFP-SPD-2 was used to validate a failure in centrosome duplication; a control GFP-PAR-2; GFP-SPD-2 embryo is shown for comparison. Solid green lines, PAR-2 boundaries; dotted green lines, cortical PAR-2; blue 'x', pronuclei. Embryo posterior is to the right. Anterior (Ant.) and posterior (Post.) are indicated on the graphs; all extensions are shown as fractions of the embryo length. Scale bars, 10 µm. In the *zyg-1(b1ts)paternal* experiment shown, the kymograph corresponds to approximately eleven minutes during polarity establishment although the recording continued through cell division. Only one centriole is contributed by *zyg-1(b1ts)* sperm[2]; these embryos form a monopolar spindle at the first mitosis (25:35). *sas-4(RNAi)* prevents duplication during the first cell cycle[3,4], leading to a single centrosome at the two-cell stage (39:30). Defects in DNA replication lead to a significant cell cycle delay[5,6], also seen in *mcm-4(RNAi)* embryos.

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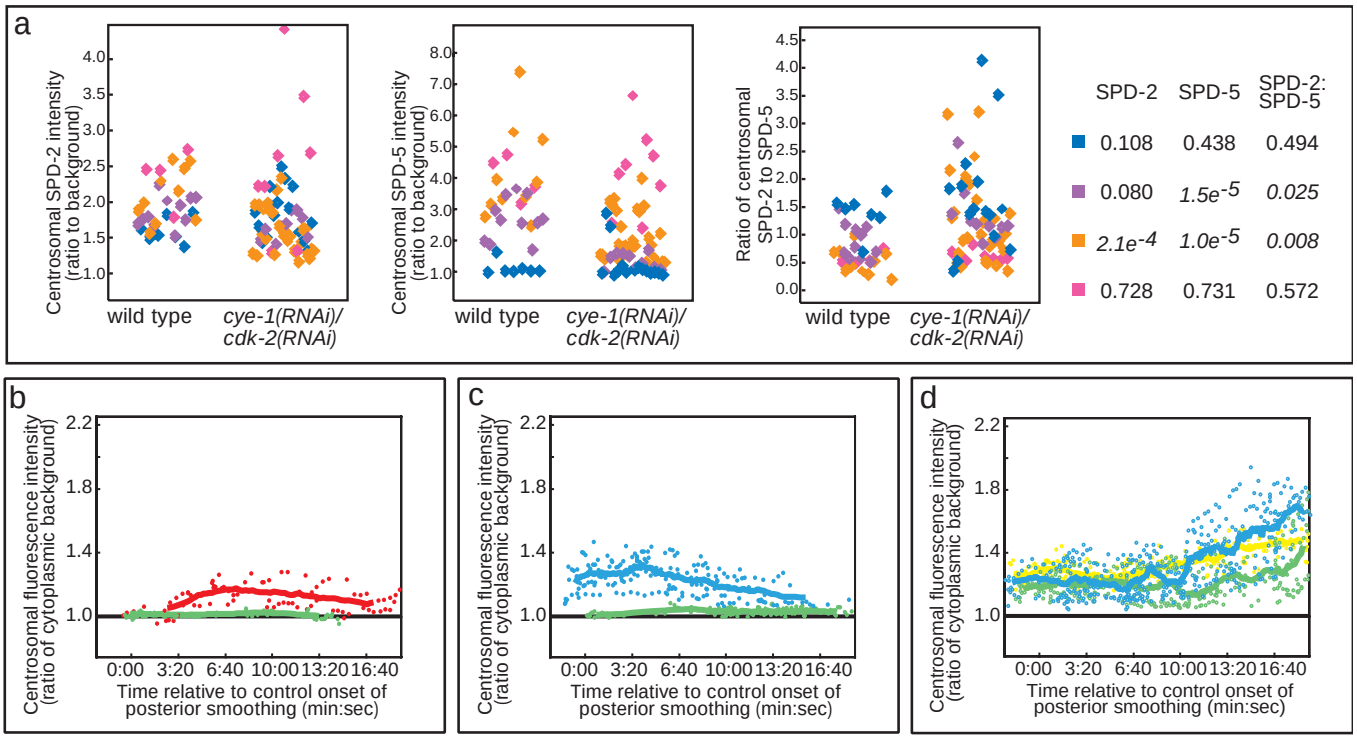

Figure S3 (a) Quantification of centrosomal SPD-2 and SPD-5. Levels were calculated from immunofluorescence images, as shown in Fig. 3. Embryos were sorted into four temporal stages based on chromatin morphology and nuclear size (see Materials and Methods) representing meiosis (dark blue), early prophase (polarity initiation – pronuclear migration; purple), late prophase (pronuclear meeting – NEBD; orange), and mitosis (prometaphase, metaphase, anaphase; pink). Each dot represents one centrosome. The temporally-grouped centrosomal intensities were tested for sameness between wild type and *cye-1(RNAi)/cdk-2(RNAi)* embryos. P values are listed in the Table ; $P < 0.050$ was considered significant, indicated with italics. **(b-c)** SPD-2 and SPD-5 are required for centrosome assembly throughout the cell cycle. Recruitment of the PCM proteins SPD-2 (blue), AIR-1 (green), and γ–tubulin (red) in *spd-2(RNAi)* and *spd-5(RNAi)* embryos. Centrosomal GFP-γ-tubulin was not detectable in *spd-5(RNAi)* embryos, in agreement with previous data [1]. *spd-2*, *spd-5*, and *air-1* are required for polarity establishment but γ–tubulin is not [1-4]. **(d)** Cyclin E-Cdk2 and AIR-1 are required independently for centrosomal SPD-2 recruitment. Accumulation of SPD-2 in *cdk-2(RNAi)* (blue), *air-1(RNAi)* (yellow), and *cdk-2+air-1(RNAi)* (green) embryos.

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Movie Legend

Supplementary Information Movie S1. Composite movie of PAR-2 and contractile polarity in control, *cye-1(RNAi)*, and *cdk-2(RNAi)* embryos. GFP-PAR-2 and DIC time-lapse images were recorded. The posterior is to the right in the control embryo; the meiotic pole is to the left in the *cye-1(RNAi)* and *cdk-2(RNAi)* embryos. The individual movies were recorded under similar conditions and play at the same speed, roughly 150X real-time. All movies start at a similar time: in the control embryo, the onset of posterior smoothing; in the *cye-1(RNAi)* and *cdk-2(RNAi)* embryos, a similar cell cycle stage judged by the size of the male pronucleus and the time elapsed from meiosis II. The movies stop at the completion of cytokinesis. In the *cdk-2(RNAi)* embryo, a patch of PAR-2 appears on the cortex after roughly thirteen minutes. There is a noticeable cell cycle delay in the *cye-1(RNAi)* embryo.

Supplementary Information Movie S2. Composite movie of GFP-PAR-2 polarity following depletion of essential cyclins. Embryos are shown with posterior to the right and, in the case of *cye-1(RNAi)*, with the meiotic pole to the left. The identity of each cyclin is indicated in the movie. The individual movies were recorded under similar conditions and play at the same speed, roughly 150X real-time. The movies are standardized to start at a similar cell cycle time, roughly one minute before polarity establishment. The *cye-1(RNAi)* embryo time was standardized based on the size of the male pronucleus. The movies stop about twenty minutes after polarity initiation, at the onset of cytokinesis furrow ingression in the control embryo.

Table S1. dsRNA primer sequences, clone information, RNAi method, and conditions.

	Gene	Primer 1 sequence	Primer 2 sequence	Clone number	Method	Conditions
air-1	K07C11.2	GCCTCTCGGAAAAGGAAAGT	CCTTGATTCTGGCGATCAAT		F	22 h. 25°C
cdk-2	K03E5.3			I-1D09	F	24 h. 25°C
cyb-1	ZC168.4	CGCAAGGTCTTCAGACGAA	GACAAGCTTCCCCTGTTTGA		I	17 h. 25°C
cyb-2.1	Y43E12A.1			IV-5H02	F	24 h. 25°C
cyb-2.2	H31G24.4	GGAGCTCCGTCAAGAATCAA	CGCAAAGCTTCTGATGTCTG		I	17 h. 25°C
cyb-3	T06E6.2	GCGCACACATCTCTGGATT	ATACTTCTGGCGAACGGATG		I	22 h. 25°C
cye-1 RNA1	C37A2.4	TATCCGGATGATTTCCAAGC	GAAGCATCGAAATGGTAGCC		I	22 h. 25°C
cye-1 RNA2	C37A2.4	GCTGGAAGAAAGT	CTTAGTACTTCGT		I	22 h. 25°C
cyh-1	Y49F6B.1	AAATCCCGATTTTCCGTACC	GTCGAGAAAACCCCAAAAGA		I	22 h. 25°C
cyl-1	C52E4.6			V-8E15	F	24 h. 25°C
mcm-4	Y39G10AR.14	GACAAGGGACGTATCGAGGA	TATTCGCTTTTGCGTAGGCT		I	22 h. 25°C
sas-4	F10E9.8	GTGAGGCTCAAACGGGAATA	TCCTGTGGTACAGCTTCCAA		I	24 h. 25°C
spd-2	F32H2.3	TGCATGCGAATAAGACGAAG	TTGCGGACACAGAAAACAAA		I	22 h. 25°C
spd-5	F56A3.4	TGTCGCAACCAGTTCTGAAT	ATGGAGGCAAATTGTTGCTG		I	22 h. 25°C
A-type cyclin	ZK507.6	GCGAAAAGTCAAGCAGTGAA	ACCAAGATGATGTGGGCATT		I	22 h. 25°C

Production of dsRNA in vitro was performed as described. The gene-specific primer sequences used are indicated in the table; either a T3 or T7 polymerase recognition site was added to the 5' end of the sequences listed. Genomic N2 DNA was used as the template for in vitro RNA production and cloning. To generate the *air-1* feeding clone, we used the primers indicated to amplify a genomic region of air-1, which was cloned into the L4440 vector and transformed into HT115 E. coli [1]. The other feeding clones used were obtained from Geneservice Ltd (Cambridge, UK), and the identification numbers are provided. RNAi was performed either by feeding worms with bacteria producing dsRNA (feeding, F) or by injection of dsRNA transcribed in vitro (injection, I). Feeding was performed as described [2]. The conditions refer to the time elapsed (hours, h.) from injection or placing worms on feeding bacteria until recording and should be considered as averages (approximately ± 1 hour). All worms were kept at 25°C. We analyzed PAR-2 polarity using two different dsRNAs for *cye-1* and the results were indistinguishable. *cye-1* RNA1 was used for subsequent experiments. Both *cyb-1(RNAi)* and *cyb-2.2(RNAi)* resulted in sterility after longer incubation times.

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