Supplementary information

Supplemental materials and methods

C. elegans strains

The following strains were used: N2 (wild type), KK184 (*par-4(it47)* V), KK822 (*par-1(zu310)* V), and VC388 (*cyb-3(gk195)* V/nT1 [qIs51] (IV;V). N2 and VC388 were maintained at 20°C, KK184 and KK822 were maintained at 15°C. For temperature shift, KK184 and KK822 young adults were incubated at 24°C for 24 hours. The VC388 strain is balanced by nT1, a reciprocal translocation between linkage groups IV and V. All fertile VC388 progeny are *cyb-3* $^{+/-}$ and these animals produce aneuploid progeny that arrest as embryos or early larvae, fertile *cyb-3*$^{+/-}$ animals, and slow-growing *cyb-3*$^{-/-}$ animals that arrest as L2-L3 larvae (W.M.M., unpublished observations). Arrested embryos and larvae of the *cyb-3*$^{+/+}$ genotype are due to the nT1 balancer translocation.

RNAi

RNAi feeding vectors: feeding vectors against *wee-1.3, atl-1, chk-1,* and *cdk-1* were obtained from the Ahringer feeding library (Source Bioscience). For *cyb-1*, a 534 nt EcoRI-XhoI fragment was isolated from the *cyb-1* cDNA and subcloned into pL4440. This fragment shows 81% identity over a 426 nt stretch to the *cyb-2.1* gene, and 82% identity over a 428 nt stretch to the *cyb-2.2* gene. Using the E-RNAi off-target evaluation software (www.e-rnai.org) it was determined that this RNAi feeding vector could generate 28 21-nt siRNAs against *cyb-2.1* and 24 21-nt siRNAs against *cyb-2.2*, and thus co-depletion of CYB-2.1 and CYB-2.2 with CYB-1 is considered likely. For *cyb-3,* a 1131 nt fragment corresponding to the entirety of the *cyb-3* open reading frame was subcloned into pL4440 as an EcoRI-SalI fragment. This fragment showed no significant identity to any sequences beyond *cyb-3* in the *C. elegans* genome, as inferred by BLAST searches performed on the NCBI database, and no off-target hits were identified using E-RNAi. The resulting plasmids were then transformed into bacterial strain HT115(DE3).

RNAi conditions: all RNAi was done by feeding and incubation was at 21°C. Standard nematode growth media plates were supplemented with 0.5 mM IPTG and 100 ug/ml carbenicillin. Plates were seeded with overnight cultures of HT115(DE3) transformed with the appropriate feeding vector, as described below, and allowed to dry overnight. Plates were used the following day. For *cyb-1* RNAi, L1-stage animals were plated on *cyb-1* RNAi bacteria and incubated for 60 hours prior to embryo isolation. Under these conditions we observed a high level of embryonic lethality (Fig. S2A), illustrating the efficacy of the RNAi. For *cyb-3* RNAi, L4-stage animals were plated on a mixture of 10% *cyb-3* RNAi bacteria and 90% vector-only (pL4440) bacteria and incubated for 24 hours prior to embryo isolation. For *atl-1/chk-1* RNAi, L1-stage animals were plated on 3:1 mixture of *atl-1* RNAi bacteria and *chk-1* RNAi bacteria and incubated for 36 hours. Animals were then transferred to plates containing 100% vector-only bacteria for an additional 24 hours prior to embryo isolation and timing. For *wee-1.3* RNAi, young adult animals were plated on a mixture of 10% *wee-1.3* RNAi bacteria and 90% vector-only bacteria and incubated for 24 hours prior to embryo isolation and timing. If the time spent on RNAi food was increased, or if the amount of *wee-1.3* RNAi bacteria on the plates was increased, then few if any embryos were produced, owing to defects in oocyte maturation (Burrows et al., 2006). For *atl-1/chk-1/cyb-3* RNAi, L1-stage animals were plated on 3:1 mixture of *atl-1* RNAi bacteria and *chk-1* RNAi bacteria and incubated for 36 hours. Animals were then transferred to plates containing 90% vector-only bacteria and 10% *cyb-3* bacteria for an additional 24 hours prior to embryo isolation and timing.

Antibodies

Mabs F2F4 and K76 were obtained from the Developmental Studies Hybridoma Bank (University of Iowa) and were used at 1:50. P-CDK-1 was detected using antibody sc-7989R (Santa Cruz Biotechnology) diluted at 1:50. PCN-1 was detected using a commercially supplied (Bethyl Laboratories), affinity-purified rabbit antibody raised against a synthetic peptide corresponding to the amino acid sequence N-DIDSEHLGIPDQDYAVVCE-C of *C. elegans* PCN-1.

Antibody validation

Anti-PCN-1: this antibody recognized a single band of the appropriate size on Western blots of embryo extracts (Fig. S1A).

MabF2F4: Mab F2F4 did not detect a band of any size on Western blots of *C. elegans* embryo extracts (data not shown), suggesting that its antigen in *C. elegans* is not recognized when denatured. Therefore, RNAi was used to deplete embryos of either *cyb-1* or *cyb-3* followed by MabF2F4 staining (Fig. S2B), and this revealed that the MabF2F4 antigen was specifically depleted by *cyb-3* RNAi. As detailed above, the *cyb-1* RNAi used here is likely to also co-deplete *cyb-2.1* and *cyb-2.2*, and thus the signal observed after Mab F2F4 staining of *cyb-1(RNAi)* embryos suggests that Mab F2F4 does not efficiently recognize CYB-1, CYB-2.1, or CYB-2.2. RNAi against both *cyb-1* and *cyb-3* was effective, as evidenced by a high level of embryonic lethality after depletion of either gene (Fig. S2A). To confirm Mab F2F4 cross-reactivity with CYB-3, L2 larvae from strain VC388 were fixed and stained with Mab F2F4 and Mab K76, which recognizes P-granules, and the samples were genotyped by the presence (*cyb-3*$^{+/-}$) or absence (*cyb-3*$^{-/-}$) of pharyngeal GFP signal. As shown in Fig. S2C, *cyb-3*$^{+/-}$ animals displayed a strong Mab F2F4 signal in the developing germline and also in some surrounding somatic nuclei. By contrast, *cyb-3*$^{-/-}$ animals contained just two germ cells, and these were devoid of Mab F2F4 signal (Fig. S2C). Based on the data in Fig. S2, we conclude that Mab F2F4 is specific for CYB-3.

Anti-P-CDK-1: this antibody recognized a single band of the appropriate size on Western blots of embryo extracts (Fig. S3A), and furthermore, anti-P-CDK-1 produced a nuclear signal in control RNAi embryos that was greatly diminished in both *cdk-1(RNAi)* and *wee-1.3(RNAi)* embryos (Fig. S3B).

Immunostaining and image analysis

Antibody staining: embryos were isolated for staining by bleaching young gravid adults. Embryos were spotted on poly-L-lysine coated slides, a coverslip was applied, and the samples were then frozen on dry ice for 10 minutes. The coverslip was then flicked off and the samples processed in one of two ways. For PCN-1, the

slides were submerged in -20° methanol for one minute. Slides were then transferred to fix solution (1X PBS, 80mM HEPES pH 6.9, 1.6 mM $MgSO_4$, 0.8 mM EGTA, 3.65% formaldehyde) at room temperature for 20 minutes, washed three times in TBS with 0.1% Tween-20 (TBS-T), and blocked for one hour in goat serum. Primary antibodies were applied for either overnight incubation at 4°C or room temperature incubation for two hours. Slides were then washed three times with TBS-T, secondary antibodies were applied, and incubation was carried out at room temperature for 1-2 hours. Slides were then washed three times with TBS-T, mounted in a solution containing DAPI stain, and coverslips were applied and sealed. For MabF2F4 and P-CDK-1 staining, the slides were submerged in -20° methanol for 15 minutes, and then rehydrated in PBS for 5 minutes. Primary antibodies were applied (in PBS) and incubated for 45 minutes at room temperature in a humid box. Slides were washed for 5 minutes in PBS with 0.05% Tween-20 (PBS-T) and then 5 minutes in PBS, then incubated with secondary antibodies for 45 minutes, washed again with PBS-T and then PBS for 5 minutes each, and then mounted in a solution containing DAPI stain, and coverslips were applied and sealed.

Imaging: samples were imaged on an Olympus FLUOVIEW FV1000 confocal laser-scanning microscope. Images (1024x1024 pixel dimensions) were acquired at 40μs/pixel using the 40x objective. Images were not altered after initial capture. For quantification, single optical slices corresponding to maximum CYB-3 signal intensity for AB and P_1 nuclei within a given embryo were quantified for pixel values. For each pair, the AB value was set to 100 and the P_1 value adjusted accordingly. The P_1 values were then averaged.

Supplemental references

Burrows, A.E., Sceurman, B.K., Kosinski, M.E., Richie, C.T., Sadler, P.L., Schumacher, J.M. and Golden, A. (2006). The *C. elegans* Myt1 ortholog is required for the proper timing of oocyte maturation. *Development* **133**, 697–709.

Sulston, J.E., Schierenberg, E., White, J.G and Thomson, J.N. (1983). The embryonic cell lineage of the nematode *Caenorhabditis elegans*. *Dev. Biol.* **100**, 64–119.

Figure S1

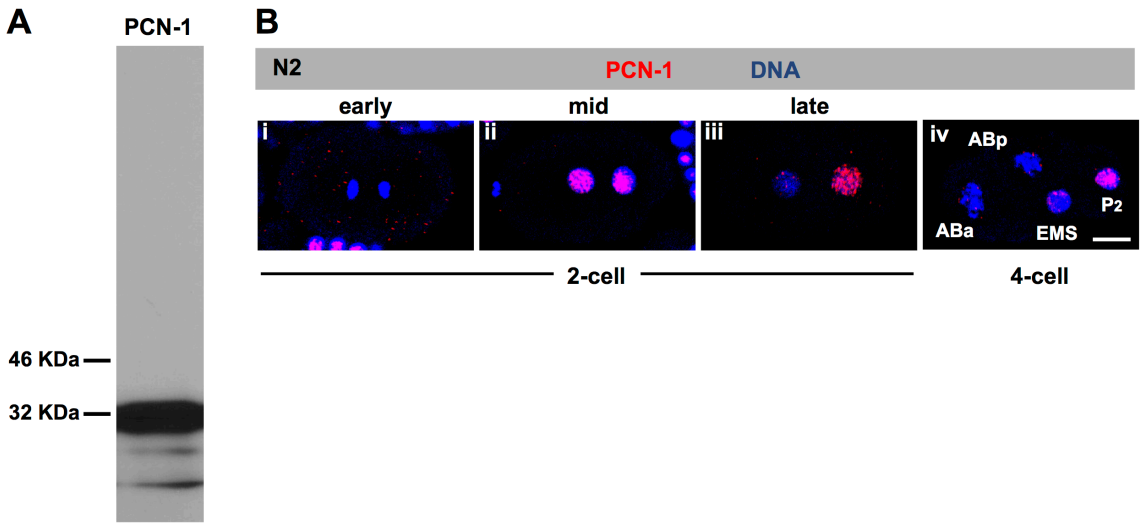


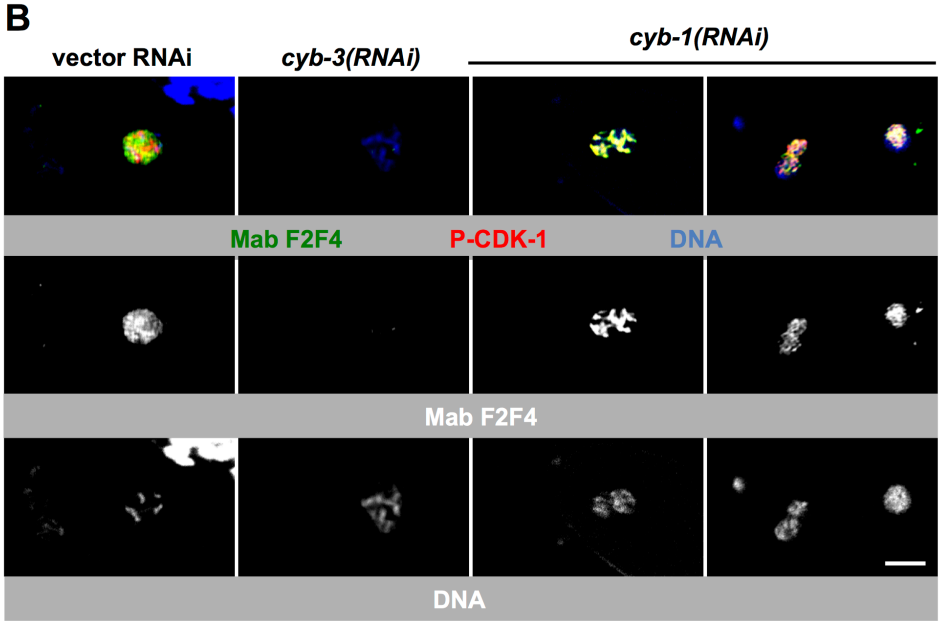
Figure S1. Validation of PCN-1 antibody and confirmation that PCN-1 staining reliably detects replicating nuclei. (A) Embryos were isolated from strain N2 by bleaching, washed, resuspended in SDS-PAGE sample buffer, boiled for 10 minutes, and then loaded on an SDS-PAGE gel. The gel was transferred and probed by Western blotting with anti-PCN-1. (B) Embryos were stained exactly as in Figs 1B-C. Panel i shows an early S-phase embryo that has just begun replication and thus little PCN-1 signal has yet to accumulate. Panel ii shows a later two-cell embryo; note that the PCN-1 signal covers more of the nuclear interior in the anterior AB cell than in P_1. This is consistent with more robust replication in AB during early S-phase (Edgar and McGhee, 1988; Benkemoun et al., 2014). Panel iii shows an older two-cell embryo, where AB has nearly completed S-phase whereas P_1 is still solidly in S-phase. Panel iv shows a four-cell embryo where the P_2 cell is still in S-phase while the other cells are nearly done. This is consistent with P_2 being the last cell to divide amongst the four cells depicted (Sulston et al., 1983). Bar, 10μM.

Figure S2

A

RNAi	# eggs laid	# eggs hatched
vector	260	260
cyb-1	308	39
cyb-3	279	0

B



C

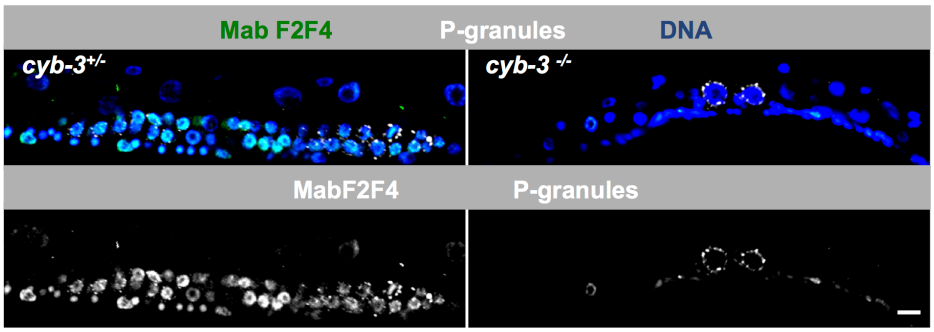


Figure S2. Validation that MabF2F4 specifically recognizes CYB-3. (A) Embryonic lethality after the indicated RNAi. Adults were allowed to lay eggs for 24 hours and then removed. The eggs were counted (# eggs laid) and then counted again 24 hours later to determine the number of eggs that hatched. (B) Animals were treated with the indicated RNAi and embryos were isolated, fixed, and stained with Mab F2F4 (green in top panels), anti-P-CDK-1 (red in top panels), and DAPI

(DNA, blue in top panels). Bottom panels show uncolored images with the Mab F2F4 and DNA signals alone. For *cyb-1(RNAi)* embryos two samples are shown, one of which shows Mab F2F4 signals in an embryo with multiple nuclei (final set of panels). Bar, 10μM. (C) Stage L2 animals of the indicated genotype were fixed and stained with Mab F2F4 (green in top panels), Mab K76 (P-granules, white in top panels), and DAPI (DNA, blue in top panels). Bottom panels show uncolored images with the Mab F2F4 and K76 signals alone. Bar, 5μM.

Figure S3

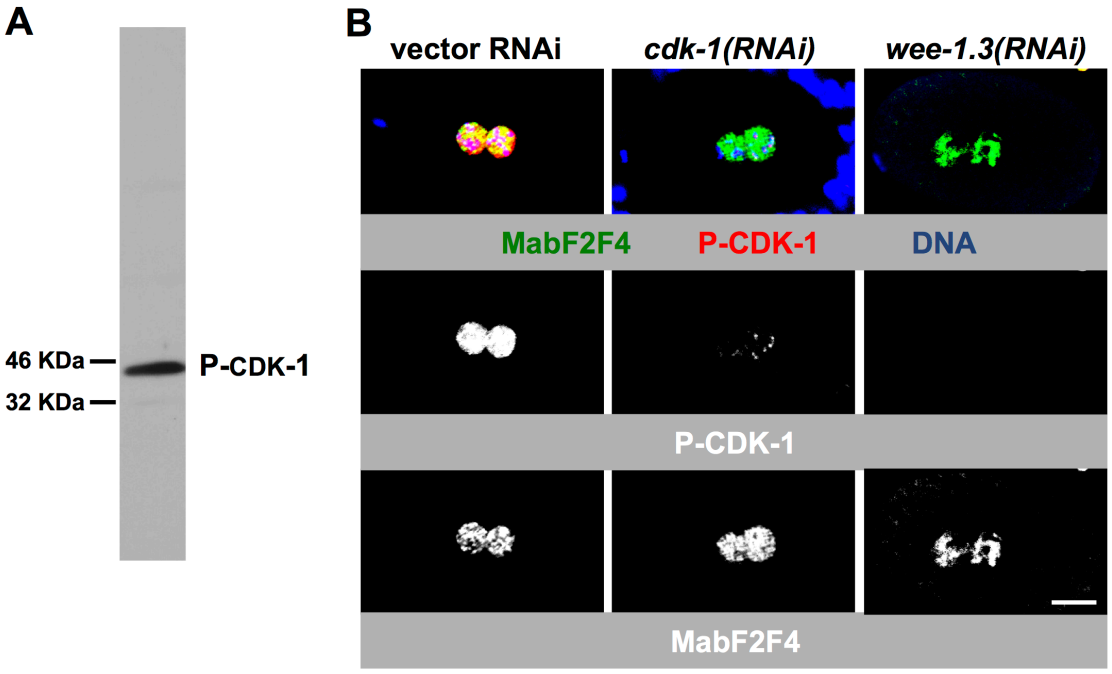


Figure S3. Validation of anti-P-CDK-1 antibody. (A) Embryos were isolated from strain N2 by bleaching, washed, resuspended in SDS-PAGE sample buffer, boiled for 10 minutes, and then loaded on an SDS-PAGE gel. The gel was transferred and probed by Western blotting with anti-P-CDK-1. (B) The indicated RNAi embryos were fixed and stained exactly as in Fig. 3. Bar, 10μM.