

## RESEARCH ARTICLE

# Cyclin CYB-3 controls both S-phase and mitosis and is asymmetrically distributed in the early *C. elegans* embryo

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**ABSTRACT**

In early *C. elegans* embryos the timing of cell division is both invariant and developmentally regulated, yet how the cell cycle is controlled in the embryo and how cell cycle timing impacts early development remain important, unanswered questions. Here, I focus on the cyclin B3 ortholog CYB-3, and show that this cyclin has the unusual property of controlling both the timely progression through S-phase and mitotic entry, suggesting that CYB-3 is both an S-phase-promoting and mitosis-promoting factor. Furthermore, I find that CYB-3 is asymmetrically distributed in the two-cell embryo, such that the somatic precursor AB cell contains ~2.5-fold more CYB-3 than its sister cell, the germline progenitor P<sub>1</sub>. CYB-3 is not only physically limited in P<sub>1</sub> but also functionally limited, and this asymmetry is controlled by the *par* polarity network. These findings highlight the importance of the CYB-3 B3-type cyclin in cell cycle regulation in the early embryo and suggest that CYB-3 asymmetry helps establish the well-documented cell cycle asynchrony that occurs during cell division within the P-lineage.

**KEY WORDS:** *Caenorhabditis elegans*, Early embryo, Cell cycle, CYB-3 cyclin, Asynchrony, DNA replication

**INTRODUCTION**

How cell cycle control pathways are regulated during early embryogenesis and the means by which cell cycle timing impacts development are important and open research questions that span the fields of cell and developmental biology. The early *C. elegans* embryo is particularly well suited to address these problems as it is amenable to both cytological and genetic analysis, and these attributes have allowed multiple large-scale efforts that have revealed the genes, protein-protein interactions and molecular machineries required for cell division in the early embryo (Fraser et al., 2000; Gönczy, et al., 2000; Kamath et al., 2003; Gunsalus et al., 2005; Sönnichsen et al., 2005; Boxem et al., 2008). Despite these advantages, however, we still only have a rudimentary understanding of how the cell cycle is regulated in the early *C. elegans* embryo and thus further efforts are required if we are to achieve a systems-level understanding of this crucial process.

The first mitotic cycle in the early embryo is initiated shortly after fertilization and the completion of meiosis. Sperm entry triggers the maternal pronucleus to complete meiotic divisions and both the maternal and paternal pronuclei then initiate DNA replication (Edgar and McGhee, 1988; Sonnevile et al., 2012). As replication

is finishing, the maternal pronucleus begins to migrate across the embryo where it eventually meets with the paternal pronucleus (pronuclear meeting, or PNM), which is positioned at the future posterior side of the embryo. The two pronuclei then move towards the embryo center in a process termed pronuclear centration (PNC), where they remain prior to nuclear envelope breakdown (NEB) and entry into mitosis. Interphase of the first cell cycle can thus be divided into two portions: S-phase takes about half the time and is complete by the time of PNM (Sonneville et al., 2012, 2015); and the second half is composed of a G<sub>2</sub>/prophase period when nuclei display highly condensed chromosomes and an intact nuclear envelope. Key developmental events are also occurring during the first cell cycle as the embryo becomes polarized along its anterior-posterior axis. Polarization is directed by the *par* network and results in asymmetric cleavage, whereby the zygote divides to form the larger AB and smaller P<sub>1</sub> cell (reviewed by Rose and Gönczy, 2014).

Polarization of the zygote produces numerous asymmetries in the early embryo that are important for both cell fate acquisition and cell cycle remodeling after the first division. For example, polarity promotes the asymmetric distribution of germ cell determinants to the germline precursor P<sub>1</sub> cell and their clearance from the somatic AB cell (Rose and Gönczy, 2014). Polarity also promotes cell cycle remodeling – the G<sub>2</sub> phase is lost in both AB and P<sub>1</sub> as these cells lack gap phases altogether and transit directly into S-phase and then mitosis (Edgar and McGhee, 1988). In addition, AB and P<sub>1</sub> inherit different cell cycle timing programs; the P<sub>1</sub> S-phase is invariably 2 min longer than the AB S-phase (reviewed by Tavernier et al., 2015). This S-phase asynchrony continues, and is extended, within the P-lineage, as the P<sub>1</sub> descendants P<sub>2</sub>, P<sub>3</sub> and P<sub>4</sub> all have progressively longer S-phases than their somatic sister cells. S-phase asynchrony is controlled by the *par* network and can be partially uncoupled from asymmetric cleavage, as mutations in two genes, *par-1* and *par-4*, retain asymmetric cleavages while S-phase asynchrony is lost (Kemphues et al., 1988; Morton et al., 1992). Whether S-phase asynchrony in P-cells is important for germline specification is not known.

Cdk-cyclin complexes provide cells with both S-phase-promoting factor (SPF) and mitosis-promoting factor (MPF) activities. In metazoans, SPFs are typically composed of Cdk2 bound to either cyclins E or A, while MPFs are Cdk1 bound to A- and B-type cyclins (reviewed by Fisher, 2011; Siddiqui et al., 2013; Wieser and Pines, 2015). In some embryonic systems, however, it might be the case that both SPF and MPF contain Cdk1 (Farrell et al., 2012), and Cdk1 can perform both functions in mammalian cells that have lost Cdk2 (Ortega et al., 2003). In *C. elegans* embryos, the SPF is not known and MPF activity is provided by CDK-1 bound to either CYB-1 (a cyclin B) or CYB-3 (a cyclin B3) (van der Voet et al., 2009). CYB-1 and CYB-3 have both redundant and distinct functions during the early cell cycles. Depletion of CYB-1 causes a modest delay in NEB and a profound defect in

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chromosome congression to the metaphase plate (van der Voet et al., 2009). Depletion of CYB-3 causes a more severe delay in NEB, and cells then arrest at metaphase (Cowan and Hyman, 2006; van der Voet et al., 2009; Deyter et al., 2010). Thus, as is the case for cyclin B3 proteins in other organisms (Yuan and O'Farrell, 2015; Zhang et al., 2015), CYB-3 is required to promote anaphase. Interestingly, work in *C. elegans* has revealed additional functions for CYB-3 prior to mitotic entry (Deyter et al., 2010). In *cyb-3(RNAi)* zygotes, interphase events such as chromosome condensation, pronuclear migration and centrosome maturation are all delayed, suggesting that CYB-3 has important interphase functions in addition to a role in mitosis. To date, CYB-1 and CYB-3 are the only cyclins known to have a major role in cell cycle progression in the early embryo. Two other cyclin B orthologs, the highly related CYB-2.1 and CYB-2.2 proteins, are not essential for viability nor are they required for proper cell cycle timing in early embryos (Rabilotta et al., 2015). Loss of a cyclin A ortholog, CYA-1, causes late embryonic lethality whereas early embryogenesis occurs normally (Yan et al., 2013). Furthermore, previous work has examined loss of *cdk-2* and *cye-1* (cyclin E) in the early embryo (Cowan and Hyman, 2006), and although a minor cell cycle delay was observed there were no obvious defects in either DNA replication or centrosome duplication.

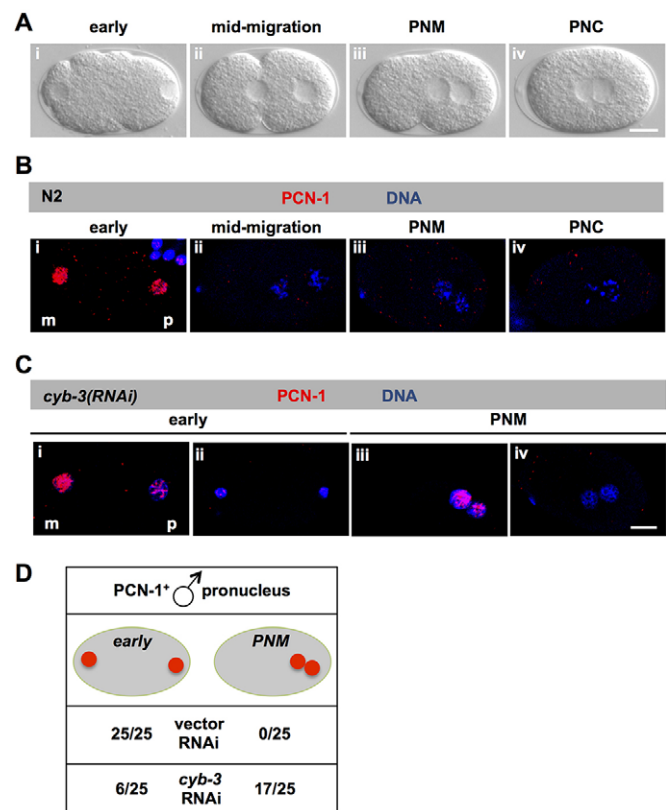
In this study I further explore CYB-3 function during the early cell cycles in *C. elegans* embryos. I observe that loss of CYB-3 causes delays in both S-phase progression and mitotic entry in one-cell embryos, suggesting that this single cyclin might contain both SPF and MPF activities. In addition, I report that at the two-cell stage CYB-3 is asymmetrically distributed in a *par*-dependent manner, such that AB contains ~2.5-fold more CYB-3 than does P<sub>1</sub>. Furthermore, CYB-3 is not only physically limited in P<sub>1</sub> relative to AB, but is also functionally limited. These data provide new insight into how the cell cycle is regulated during early embryogenesis and also suggest a new model for how S-phase asynchrony is achieved in the early embryo.

## RESULTS

### CYB-3 is required for timely DNA replication

Previous findings have linked CYB-3 to DNA replication in *C. elegans* embryos. For example, it was recently shown that chromosome condensation is dependent on DNA replication (Sonneville et al., 2015), and previous work has shown that condensation is delayed in *cyb-3(RNAi)* embryos (Deyter et al., 2010). In addition, the canonical metazoan SPF, Cdk2-cyclin E, is dispensable for replication in early embryos (Cowan and Hyman, 2006), suggesting that an atypical SPF is involved. I therefore asked if CYB-3 plays a role in DNA replication.

Replication was analyzed by immunostaining fixed embryos with an antibody raised against PCN-1, the worm ortholog of the replication fork protein PCNA. This antibody recognizes a single major band of the appropriate size on western blots of early embryos (Fig. S1A). Wild-type one-cell embryos were examined first, and these could be staged temporally by the position of the pronuclei (Fig. 1A). Early embryos contain well-separated pronuclei (Fig. 1Ai), in older embryos the maternal pronucleus migrates (Fig. 1Aii), then meets the paternal pronucleus (PNM; Fig. 1Aiii), and finally the two pronuclei assume the central position (PNC; Fig. 1Aiv). In wild-type embryos, a bright PCN-1 signal was observed in early embryos, where both the maternal and paternal pronuclei were intensely stained (Fig. 1Bi). In contrast to early one-cell embryos, little or no PCN-1 signal was observed in older mid-migration embryos, or in even older PNM or PNC embryos (Fig. 1Bii-iv).



**Fig. 1. CYB-3 is required for timely DNA replication in early *C. elegans* embryos.** (A) Time-lapse DIC images of a single embryo progressing through the first mitotic cycle. The developmental stage of the embryo is indicated. PNM, pronuclear meeting; PNC, pronuclear centration. (B) Early wild-type embryos were fixed and immunostained for PCN-1 (red) and stained with DAPI to visualize the DNA (blue). Panels are organized as in A. Maternal (m) and paternal (p) pronuclei are labeled in Bi. (C) Same as in B except that embryos were obtained from *cyb-3(RNAi)* mothers and two examples each of early and PNM embryos are shown. (D) Quantification of PCN-1 signal distribution in the paternal pronucleus for vector RNAi control and *cyb-3(RNAi)* samples. Data shown are from a single experiment and are representative of two independent biological replicates. Scale bars: 10  $\mu$ m.

Importantly, previous work had examined replication kinetics in one-cell embryos using live cell imaging and a GFP-tagged CDC-45 protein, a marker for the presence of active replication forks (Sonneville et al., 2012, 2015). This work demonstrated that replication initiates in both pronuclei very shortly after the completion of meiosis, and that replication is complete prior to the PNM stage. Data shown in Fig. 1B are in excellent agreement with these previous studies. Furthermore, as detailed in Fig. S1B, PCN-1 staining of two- and four-cell embryos also gave the expected pattern whereby nuclei are PCN-1 positive only if they are in S-phase. Based on the data in Fig. 1B and Fig. S1B, I conclude that PCN-1 immunostaining is a reliable method to mark actively replicating nuclei in early embryos.

To examine the impact of CYB-3 depletion on DNA replication, I used RNAi to reduce CYB-3 levels. As detailed in the supplementary Materials and Methods, our RNAi targeting construct is expected to be specific for *cyb-3* and not co-deplete other *cyb* gene products. The RNAi conditions were effective, as assessed by the high (100%) embryonic lethality in the progeny of fed mothers (Fig. S2A). To examine replication, control or *cyb-3(RNAi)* embryos were fixed and stained for PCN-1 (Fig. 1C,D). The control RNAi samples were highly similar to the samples shown in Fig. 1B, as expected

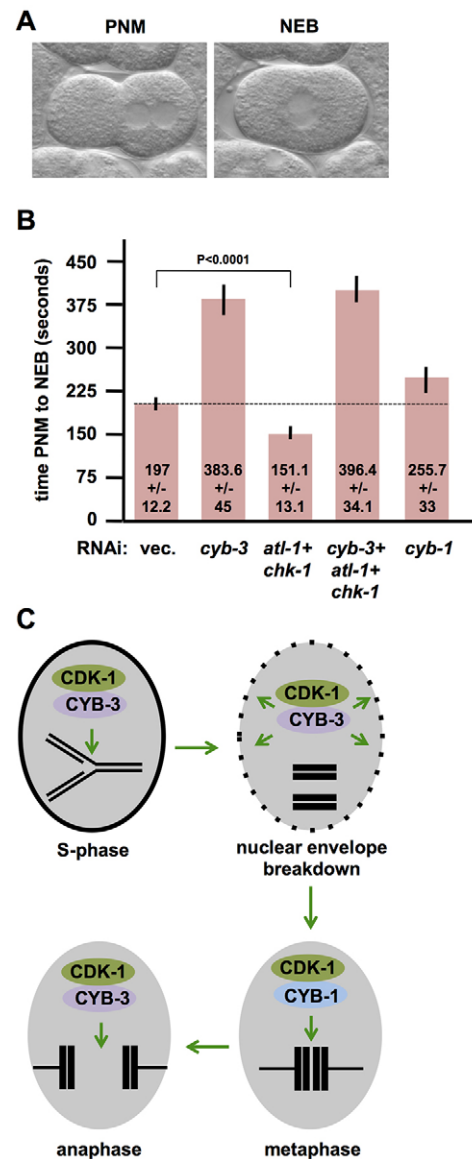
(summarized in Fig. 1D). By contrast, *cyb-3(RNAi)* embryos revealed a very different pattern. In many embryos, there was as an imbalance between the maternal and paternal pronuclei, such that the PCN-1 signal was more intense in the maternal pronucleus (Fig. 1Ci).

Because CYB-3 is required for the proper completion of meiotic anaphase II, polar body extrusion often fails in *cyb-3(RNAi)* embryos (van der Voet et al., 2009; Deyter et al., 2010), and this can result in diploid maternal pronuclei. Thus, from embryo to embryo, *cyb-3 RNAi* produces maternal pronuclei of varying DNA content, and this complicates the analysis of replication. To avoid this I focused solely on the PCN-1 signal status of the paternal pronucleus, the DNA content of which is unlikely to be affected by *cyb-3 RNAi*. Embryos were scored for the presence or absence of PCN-1 signal in the paternal pronucleus of early embryos. Results showed that 6 out of 25 early embryos displayed a PCN-1 signal in the paternal pronucleus, but it was often weak (Fig. 1Ci,D). Importantly, the majority of early embryos (19/25) displayed no detectable PCN-1 signal in the paternal pronucleus, and these embryos often lacked a signal in the maternal pronucleus as well (Fig. 1Cii,D). This was in contrast to wild-type samples, where PCN-1 signal was always observed in early embryos (Fig. 1D). Older *cyb-3(RNAi)* embryos, at the PNM stage, were also examined, and in these the PCN-1 signal was present in the majority (17/25) of the samples examined (Fig. 1Ciii,iv,D). PCN-1-positive PNM embryos were not observed in the wild type (Fig. 1D).

These data show that DNA replication is delayed after CYB-3 depletion: replication does not occur efficiently in early samples but is observed in older samples, whereas wild-type samples show the reciprocal pattern (Fig. 1D). I note that previous work has shown that maternal pronuclear migration is delayed in *cyb-3(RNAi)* embryos (Deyter et al., 2010), and despite this I still observe replicating nuclei at the PNM stage in these embryos. This underscores the delay in DNA synthesis that occurs in *cyb-3(RNAi)* embryos, and I thus conclude that CYB-3 is required for the timely progression through S-phase in early embryos.

Data in Fig. 1 show a DNA replication function for CYB-3. Interestingly, previous work had shown that *cyb-3(RNAi)* embryos are delayed for mitotic entry at the one-cell stage (Cowan and Hyman, 2006; van der Voet et al., 2009; Deyter et al., 2010), and this could be due to a requirement for CYB-3 in initiating mitosis (i.e. NEB), or to a checkpoint-mediated delay in mitotic entry because of problems in DNA replication, or both. To examine this more closely, I asked if co-inactivation of the replication checkpoint would affect the interphase delay imposed by depletion of *cyb-3*. Previous work has established that replication stress, as imposed by depletion of replication fork components or limitation of dNTP supplies, delays interphase in one-cell embryos and that depletion of the checkpoint components ATL-1 (ATR kinase) and CHK-1 (Chk1 kinase) reverses the delay (Brauchle et al., 2003). I measured cell cycle timing in embryos depleted of *cyb-3* and compared them with embryos that had been triply depleted of *cyb-3*, *atl-1* and *chk-1*. Timing was performed as described (Sonneville et al., 2015), whereby the elapsed time between the PNM stage and NEB was recorded for each sample (Fig. 2A). Timing of a partial cell cycle is necessary at the one-cell stage as events occurring just after fertilization are difficult to visualize with differential interference contrast (DIC) microscopy (see Brauchle et al., 2003).

As shown in Fig. 2B, *cyb-3(RNAi)* embryos were delayed for mitotic entry, relative to the control samples. By contrast, *atl-1/chk-1(RNAi)* embryos showed faster cell cycle progression relative to control samples, which is consistent with previous results (Brauchle et al., 2003) and demonstrates efficacy of the RNAi. The triply



**Fig. 2. Inactivation of *cyb-3* delays interphase progression in a replication checkpoint-independent manner.** (A) Time-lapse DIC images of a single embryo progressing from the PNM stage to NEB are shown to indicate how the timing data in B were collected. (B) Ten embryos per genotype were timed as depicted in A and the data were averaged and plotted. Samples were analyzed in groups of five, the experiment was performed twice, and the values were then combined. Error bars indicate s.d.; *P*-value determined by Student's *t*-test. (C) Model depicting the division of labor for CYB-1 and CYB-3 during the early embryonic cell cycle. In this model, CYB-3 drives the early events (DNA replication and NEB), CYB-1 controls chromosome attachment to the metaphase plate, and CYB-3 then promotes anaphase.

depleted samples resembled those in which *cyb-3* alone had been depleted (Fig. 2B), showing that loss of ATL-1/CHK-1 activity has no effect on the interphase delay in *cyb-3(RNAi)* embryos. These data show that CYB-3 directly controls mitotic entry, independent of its role in promoting timely S-phase completion.

To pursue these observations, and to gain insight into the role of CYB-1 in promoting NEB, I also timed *cyb-1(RNAi)* embryos. Data showed that CYB-1 depletion caused only a modest delay in NEB (Fig. 2B), suggesting that CYB-3 plays the dominant role in promoting NEB. The *cyb-1 RNAi* was effective, as assessed by the high degree of embryonic lethality observed (87.3%; Fig. S2A).

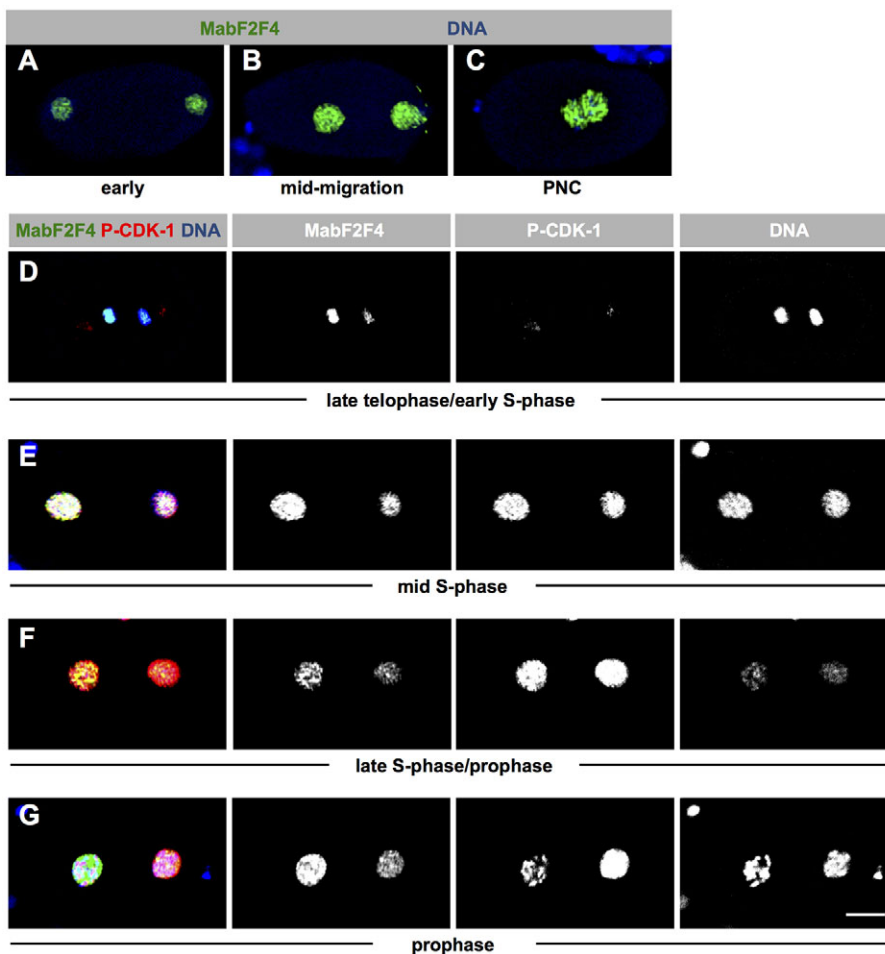
Taken together, the data shown in Figs 1 and 2 suggest a model for how CYB-1 and CYB-3 function together to mediate cell cycle progression (Fig. 2C). I propose that CYB-3 plays the major role in promoting both S-phase progression and NEB, whereas CYB-1 plays a less prominent role in these events. At metaphase, CYB-1 plays the major role in ensuring proper alignment of chromosomes to the metaphase plate (van der Voet et al., 2009), and at anaphase CYB-3 plays a major role in allowing anaphase onset (Deyter et al., 2010). As proposed by the model, our data, together with previous results, suggest that the early embryonic cell cycle in *C. elegans* is primarily driven by B-type cyclins. Although further work is needed to validate this model, it is clear that B-type cyclins assume a wider range of functions in the early worm embryo than is typically encountered in a metazoan cell cycle.

### Localization of CYB-3 in one-cell and two-cell embryos

To pursue the function of CYB-3 during S-phase in early embryos I required detailed information about its subcellular localization and intra-embryo distribution at the one- and two-cell stages. Previous work has reported an antibody specific for CYB-3 (van der Voet et al., 2009), but this antibody is no longer available. A GFP-tagged transgene was considered, but such fusions with CYB-1 have been produced and are non-functional in early embryos; more specifically, GFP-CYB-1 can be detected in oocytes and fertilized embryos undergoing meiosis but it disappears after meiosis and does not reappear during the mitotic cell cycles (Liu et al., 2004, McNally and McNally, 2005; Wang et al., 2013; W.M.M.,

unpublished observations). Given that a GFP-CYB-3 fusion would be likely to behave in a similar manner, an alternative approach was sought to localize CYB-3 within the embryo. Previous work has utilized the monoclonal antibody (Mab) F2F4, produced against *Drosophila* Cyclin B, to detect a B-type cyclin in *C. elegans* (Shakes et al., 2009; Rahman et al., 2014), although the specificity of this Mab had not yet been examined. As detailed in the supplementary Materials and Methods and Fig. S2B,C, it was determined that Mab F2F4 recognizes CYB-3. Staining of one-cell embryos with Mab F2F4 revealed a nuclear signal in early, mid-migration, and PNC embryos (Fig. 3A-C), as expected based on previous results (van der Voet et al., 2009; Rahman et al., 2014). I note that previous work using either the antibodies raised against CYB-3 (van der Voet et al., 2009) or Mab F2F4 (Rahman et al., 2014) showed a weak cytoplasmic signal in addition to a prominent nuclear signal, whereas in our experiments the cytoplasmic signal was difficult to detect whereas the nuclear signal was very strong (Fig. 3A-C). The reason for this discrepancy is unknown, but is likely to be linked to differences in the fixation protocols utilized here versus the previous studies.

I next examined CYB-3 localization at the two-cell stage. For this analysis it was also important to detect P-CDK-1, which is the tyrosine 15-phosphorylated and inhibited form of the enzyme. Tyrosine phosphorylation of Cdk-cyclin B complexes is required during S-phase to restrain mitotic entry until replication has finished (Wieser and Pines, 2015). I used a commercially available antibody raised against human P-CDK-1 for this analysis. Similar human P-



**Fig. 3. Subcellular localization of CYB-3 in early embryos.** (A-C) One-cell embryos from the indicated stages were fixed and stained with Mab F2F4 (green) and DAPI (blue) to visualize CYB-3 and DNA, respectively. (D-G) Two-cell embryos were fixed and stained for CYB-3 (Mab F2F4, green), P-CDK-1 (red) and DNA (DAPI, blue). Anterior, and thus AB, is to the left in all panels. The first column shows simultaneous detection of all three signals; the remaining columns show the individual signals. D depicts a very early two-cell embryo; E-G depict progressively later interphase embryos. Scale bar: 10 μm.

CDK-1 antibodies have been successfully used to detect P-CDK-1 in *C. elegans* embryos (Hachet et al., 2007; Rahman et al., 2014), and I found that this antibody is specific for *C. elegans* P-CDK-1 (see the supplementary Materials and Methods and Fig. S3).

Mab F2F4 and anti-P-CDK-1 were used to co-stain fixed embryos to detect CYB-3 and P-CDK-1, respectively (Fig. 3D-G). CYB-3 protein was localized to the nucleus whenever it was present, even in late telophase/early S-phase embryos (Fig. 3D). The nuclear signal persisted during S-phase and into prophase (Fig. 3E-G), which is consistent with what occurs in one-cell embryos (Fig. 3A-C). A striking feature of the CYB-3 localization pattern in two-cell embryos is that, throughout interphase, it is asymmetrically distributed such that AB contains more CYB-3 than P<sub>1</sub> (Fig. 3D-G). Importantly, this is the case even in very early two-cell embryos (Fig. 3D). This asymmetric distribution is analyzed in more detail below. Regarding the P-CDK-1 pattern, in very early two-cell embryos P-CDK-1 was not detected within nuclei (Fig. 3D), whereas in older embryos nuclear P-CDK-1 was readily detected (Fig. 3E-G). In some two-cell embryos, namely those that were in early S-phase as inferred by nuclear size and position as well as chromosome morphology, there appeared to be slightly more P-CDK-1 in AB relative to P<sub>1</sub> (see Fig. 3E). In older two-cell embryos, the P-CDK-1 signal evened out and then became more pronounced in P<sub>1</sub> relative to AB (Fig. 3F,G).

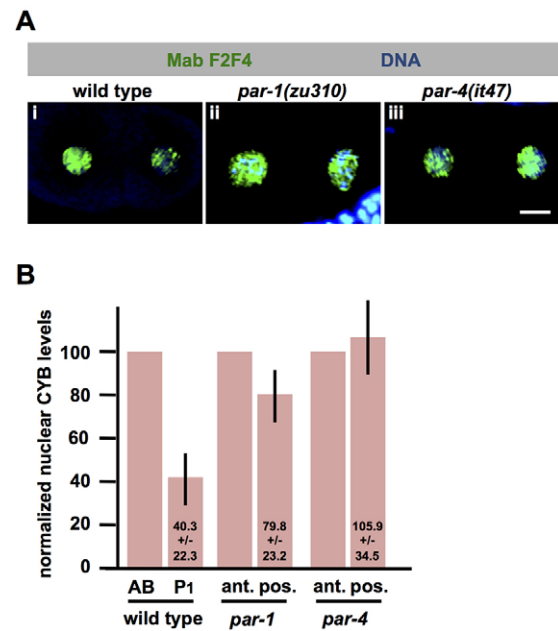
The data in Fig. 3 make two important points. First, CYB-3 is asymmetrically distributed in two-cell embryos. Second, CYB-3 protein is present in nuclei during late telophase/early S-phase, whereas P-CDK-1 is not (Fig. 3D). This suggests that CDK-1–CYB-3 complexes are active in very early S-phase, and then inactivated via inhibitory phosphorylation at some point later in S-phase, and thus that a window of opportunity exists during which CDK-1–CYB-3 can promote S-phase prior to inhibition. It is not yet known if the CYB-3 present during late telophase/early S-phase represents newly synthesized protein or protein that escaped degradation during the preceding mitosis.

### PAR-1 and PAR-4 are required for the asymmetric distribution of CYB-3

Data shown thus far reveal that CYB-3 is less abundant in P<sub>1</sub> relative to AB (Fig. 3). I next determined if this asymmetric distribution of CYB-3 is under genetic control. *par-1* and *par-4* were examined since both control S-phase asynchrony in two-cell embryos (Kemphues et al., 1988; Benkemoun et al., 2014). Consistent with Fig. 3, CYB-3 was asymmetrically distributed in wild-type two-cell embryos (Fig. 4Ai), with ~2.5-fold more CYB-3 in AB than P<sub>1</sub> (Fig. 4B). Importantly, the asymmetric distribution was compromised in *par-1* and *par-4* mutant embryos (Fig. 4Aii,iii,B). CYB-3 was evenly distributed in *par-4* mutants and the imbalance in CYB-3 levels between AB and P<sub>1</sub> was greatly diminished in *par-1* mutants. These data show that the asymmetric distribution of CYB-3 is likely to be controlled by polarity cues dependent on *par* genes, as is S-phase asynchrony.

### CYB-3 activity is limiting in P<sub>1</sub> relative to AB

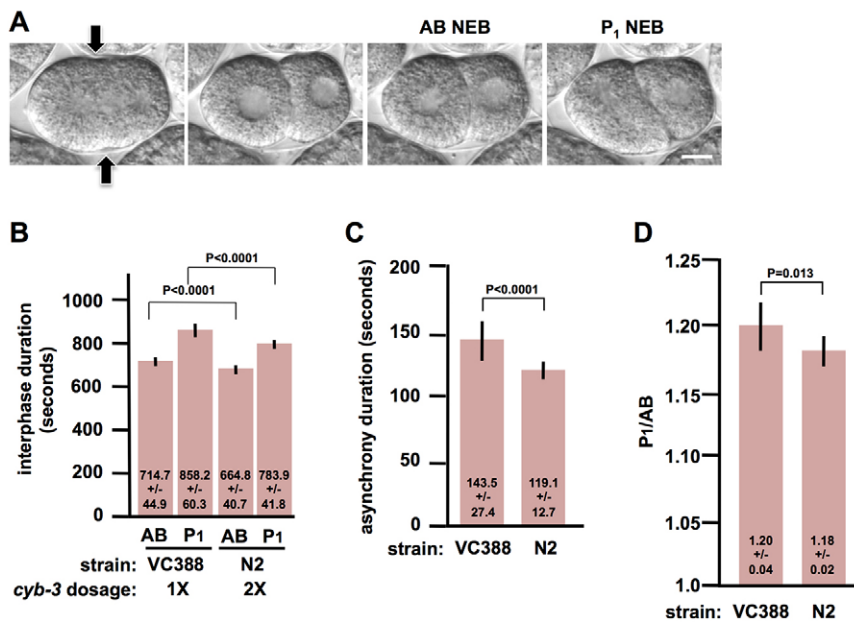
Data shown thus far indicate that CYB-3 promotes timely DNA replication and is asymmetrically distributed in two-cell embryos, such that the cell that finishes S-phase first (AB) contains more CYB-3 than its slower sister cell P<sub>1</sub>. These data suggest that CYB-3 function might be limiting in P<sub>1</sub>, relative to AB, under normal conditions. To pursue this possibility, I reasoned that if CYB-3 is limiting in P<sub>1</sub> then a reduction in CYB-3 levels should increase cell cycle timing in two-cell embryos in a manner whereby P<sub>1</sub> is



**Fig. 4. The asymmetric distribution of CYB-3 is controlled by *par-1* and *par-4*.** (A) Two-cell embryos of the indicated genotype were fixed and stained as in Fig. 3. Anterior is to the left. Scale bar: 10  $\mu$ m. (B) Quantification of CYB-3 signal in two-cell embryos. Images captured from the experiment shown in A were quantified as described in the supplementary Materials and Methods. For each AB-P<sub>1</sub> pair, pixel intensity for the AB sample was set to 100 and P<sub>1</sub> values were adjusted accordingly. Data are derived from ten embryos per genotype; error bars indicate s.d. Data shown are from a single experiment and are representative of two independent biological replicates.

impacted more than AB. I avoided using RNAi to reduce CYB-3 levels, as problems occurring prior to the two-cell stage would make the results difficult to interpret. Therefore, AB and P<sub>1</sub> timing was measured in embryos derived from mothers containing different copy numbers of the *cyb-3* gene. In two-cell embryos there is little if any zygotic transcription (Edgar et al., 1994), and thus all CYB-3 protein is derived from maternal sources. Accordingly, one would expect that the maternal *cyb-3* gene dosage would have direct effects on early embryonic cell cycle progression, as has been reported in *Drosophila* (Ji et al., 2004). The strains utilized for this analysis contain one (VC388; *cyb-3*<sup>+/-</sup>) or two (N2; *cyb-3*<sup>+/+</sup>) functional copies of the *cyb-3* gene. For VC388, only *cyb-3*<sup>+/-</sup> animals are fertile (see the supplementary Materials and Methods), and thus all embryos derived from VC388 mothers are 1X *cyb-3* with respect to the maternal contribution, whereas the wild-type N2 strain is 2X. Importantly, despite the reduction in *cyb-3* copy number, *cyb-3*<sup>+/-</sup> embryos derived from *cyb-3*<sup>+/-</sup> mothers are viable and fertile. Embryos were timed as described (Benkemoun et al., 2014), from the beginning of cortical ingression [arrows in Fig. 5A; this marks the onset of cytokinesis and telophase, which coincides with S-phase entry (Edgar and McGhee, 1988)] through to NEB in AB and P<sub>1</sub> (Fig. 5A).

Timing experiments revealed that both AB and P<sub>1</sub> cell cycles were significantly slower for VC388 than for N2 (Fig. 5B). Importantly, P<sub>1</sub>-AB asynchrony was also significantly extended in VC388 embryos (Fig. 5C), from the typical ~120 s to nearly 150 s. These data make it clear that even a modest reduction in CYB-3 levels can impact asynchrony at the two-cell stage. To determine if P<sub>1</sub> is more affected than AB by the reduction of CYB-3, I calculated the P<sub>1</sub>/AB ratio (Fig. 5D). If the ratio increases then P<sub>1</sub> is impacted more than AB, whereas if the ratio decreases then the opposite is true



**Fig. 5. CYB-3 activity is limiting in P<sub>1</sub> relative to AB.** (A) Time-lapse DIC images of a single two-cell embryo progressing through the AB and P<sub>1</sub> cell cycles are shown to indicate how the timing data in B were collected. Arrows indicate cortical ingression, which is when timing was initiated. Timing was terminated at NEB. Scale bar: 10 μm. (B) Embryos derived from the indicated strain and of the indicated *cyb-3* copy number were timed for interphase duration. Data were collected from 30 N2 embryos and 31 VC388 embryos. Samples were analyzed in groups of 10 or 11, the experiment was performed three times, and the values were then combined. (C) Asynchrony determined for the samples shown in B. (D) The P<sub>1</sub>/AB ratio for the samples shown in B. (B–D) Error bars indicate s.d.; *P*-values were determined by Student's *t*-test.

(Brauchle et al., 2003). For wild type, the P<sub>1</sub>/AB ratio is 1.18, whereas for VC388 this ratio increased to 1.20 and the difference is significant ( $P=0.013$ , Student's *t*-test). These data show that a reduction in CYB-3 levels impacts P<sub>1</sub> more than AB, and thus that CYB-3 is both physically and functionally limiting in P<sub>1</sub> relative to AB.

#### The role of inhibitory phosphorylation of CDK-1 in cell cycle asynchrony

CDK-1–CYB-3 is required for S-phase progression (Fig. 1) and, independently, mitotic entry (Fig. 2). I have also shown that CYB-3 is both physically and functionally limiting in P<sub>1</sub> relative to AB (Figs 3–5). Thus, the CYB-3 asymmetry reported here could be a trigger for cell cycle asynchrony and, if so, it could be through either its presumptive SPF or MPF functions. I reasoned that if the SPF activity were responsible, then P-CDK-1 would play an important role in maintaining asynchrony, as this is the modification that allows differential DNA replication kinetics to be translated into differential cell cycle timing. By contrast, if the MPF activity were responsible, then the P-CDK-1 modification would be dispensable, because differential MPF levels would be expected to trigger differential mitotic entry, with or without the capacity to inhibit CDK-1 during replication. I therefore investigated the impact of P-CDK-1 on asynchrony in two-cell embryos.

Upon formation of Cdk1-cyclin B complexes, the catalytic Cdk1 subunit is phosphorylated and inhibited by Wee1/Myt1 (Wieser and Pines, 2015). Indeed, it is Wee1/Myt1-mediated phosphorylation of Cdk1 that is reinforced by the replication checkpoint during S-phase so that mitosis always follows a complete round of DNA replication (Wieser and Pines, 2015). I used RNAi to deplete the *Myt1* ortholog *wee-1.3*. Timing of these embryos revealed that interphase in both AB and P<sub>1</sub> was accelerated (Fig. 6A), and that P<sub>1</sub>–AB asynchrony was reduced from ~120 s to ~65 s (Fig. 6B). This is a similar pattern to that previously observed for *atl-1/chk-1(RNAi)* embryos (Brauchle et al., 2003), and indeed I also observed accelerated interphase and reduced asynchrony after *atl-1/chk-1* RNAi (Fig. 6A,B). I next performed RNAi against all three genes, and observed in *wee-1.3/atl-1/chk-1(RNAi)* embryos that interphase acceleration was increased even further, and P<sub>1</sub>–AB asynchrony was

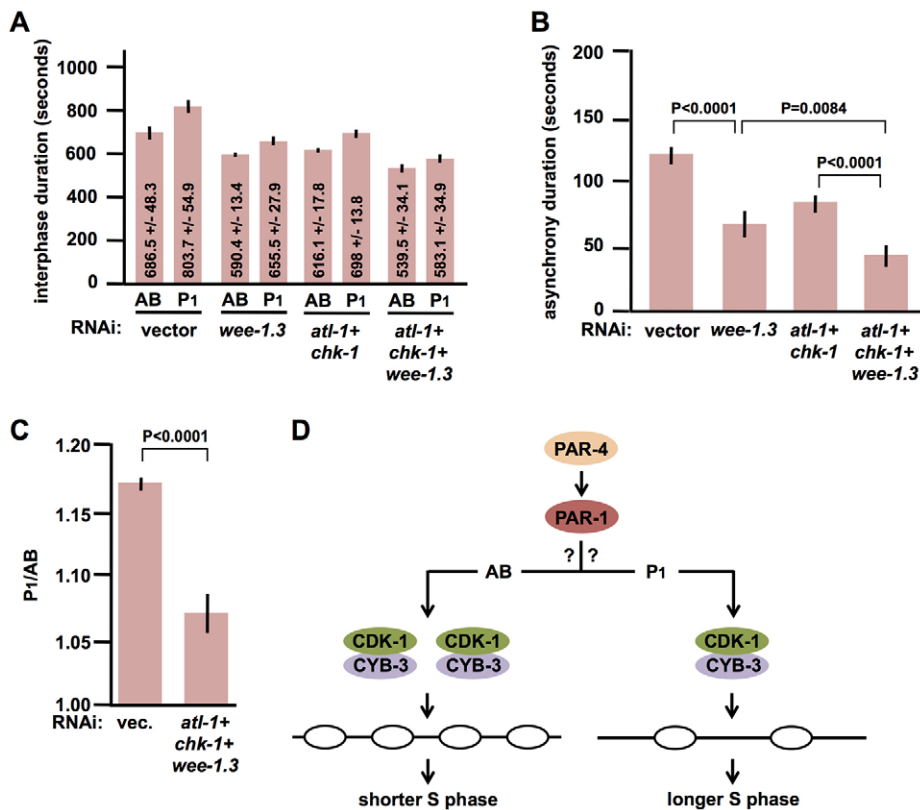
reduced even further, relative to the individual knockdowns (Fig. 6A,B). In these triply depleted embryos asynchrony was reduced to ~43 s, which is a 64% reduction relative to wild type, and the P<sub>1</sub>/AB ratio was also dramatically reduced from 1.17 to 1.08 (Fig. 6C). Based on these data, I conclude that inhibitory phosphorylation of CDK-1–CYB-3 plays a major role in maintaining cell cycle asynchrony at the two-cell stage.

#### DISCUSSION

##### Multiple roles for CYB-3 in early embryonic cell cycle progression

Work presented here, together with previous results, highlight the importance of the B3-type cyclin CYB-3 in early embryonic cell cycle progression in *C. elegans*. Previous work has shown that CYB-3 promotes timely mitotic entry, as assessed by time to NEB (Cowan and Hyman, 2006; van der Voet et al., 2009; Deyter et al., 2010), and I have shown here that it also promotes timely progression through S-phase (Fig. 1). Furthermore, I have shown that the mitotic delay observed upon CYB-3 depletion is not due to a checkpoint response to poor replication (Fig. 2), but rather reflects a direct role for CYB-3 in promoting NEB. It thus appears that CYB-3 functions continuously throughout the cell cycle, from S-phase entry to anaphase (Fig. 2C).

Our data reveal a DNA replication function for CYB-3, and this represents, to our knowledge, the first demonstration that a B3-type cyclin contains this activity. B3-type cyclins are a bit of an oddity in that, despite strong conservation of sequence and gene copy number within the metazoa, functional studies have yet to resolve a common function for the protein in cell cycle control (Lozano et al., 2012). Cyclin B3 proteins are always constitutively nuclear, but their preferred CDK binding partners show variation. Human cyclin B3 binds CDK2 but not CDK1 (Nguyen et al., 2002), the *Drosophila* protein binds CDK1 but not CDK2 (Jacobs et al., 1998), and chicken B3 binds both CDK1 and CDK2 (Gallant and Nigg, 1994). Worm CYB-3 binds CDK-1 (van der Voet et al., 2009), but whether it also binds CDK-2 has not been examined. In *Drosophila*, Cyclin B3 functions redundantly with Cyclin B during mitotic cycles and is required for female meiosis (Jacobs et al., 1998). In *C. elegans*, CYB-3 is required for both mitosis and meiosis (van der Voet et al.,



**Fig. 6. Inhibitory phosphorylation of CDK-1 is central to the establishment of asynchrony in two-cell embryos.** (A) Embryos derived from animals treated with the indicated RNAi(s) were timed for AB and P<sub>1</sub> interphase duration. Ten embryos were timed per sample set. Samples were analyzed in groups of five, the experiment was performed twice, and the values were then combined. (B) Cell cycle asynchrony determined from the data shown in A. (C) P<sub>1</sub>/AB ratio for the indicated samples. (A-C) Error bars represent s.d.; *P*-values (where shown) determined by Student's *t*-test. (D) Model of the molecular pathway for cell cycle asynchrony. In this model, PAR-4 and PAR-1 direct asymmetric distribution of CYB-3 through an unknown mechanism (as indicated by the question marks). AB receives more CYB-3 than does P<sub>1</sub>, and this in turn allows AB to initiate DNA replication more robustly, and to thereby complete S-phase before P<sub>1</sub>.

2009), while expression studies in mammals point to a meiosis-specific function (Nguyen et al., 2002). Interestingly, loss-of-function analysis has consistently revealed an anaphase function for cyclin B3, as both mitotic and meiotic cell cycles show a metaphase arrest (van der Voet et al., 2009; Deyter et al., 2010; Zhang et al., 2015); however, in some cases the metaphase arrest is dependent on the spindle assembly checkpoint (SAC) (Deyter et al., 2010) whereas in others it is not (Zhang et al., 2015). Even within *C. elegans* the exact role of CYB-3 during mitosis has been difficult to pin down. Genetic experiments support a role for CYB-3 in promoting anaphase by silencing the SAC (Deyter et al., 2010), suggesting that CYB-3 is a negative regulator of the SAC; however, other genetic studies have shown that sterility in SAC mutants is rescued by an increase in *cyb-3* gene dosage (Tarailo-Graovac et al., 2010), suggesting that CYB-3 cooperates with the SAC to ensure faithful chromosome segregation. Furthermore, loss of CYB-3 causes a SAC-dependent delay in progression to anaphase (Deyter et al., 2010), whereas overexpression of CYB-3 also causes an anaphase delay, but in a SAC-independent manner (Tarailo-Graovac and Chen, 2012). As reported here, CYB-3 is required for efficient DNA replication, and thus analysis of mitosis in samples depleted of CYB-3 is complicated by the fact that under-replicated chromosomes may be present. This raises the possibility that some mitotic phenotypes assigned to CYB-3 might be indirect effects of a failure to efficiently replicate the chromosomes in the preceding S-phase.

### Asymmetric distribution of CYB-3

Another surprising finding reported here is that CYB-3 is asymmetrically distributed, in a manner dependent on PAR-1 and PAR-4, in two-cell embryos. Interestingly, I also routinely observed that CYB-3 was asymmetrically distributed in the P<sub>1</sub> division products, such that EMS inherited more CYB-3 than its

sister P<sub>2</sub> (data not shown), which suggests that an asymmetric distribution of CYB-3 is a general feature of cell division within the P-lineage. PAR-1 and PAR-4 are members of the PAR polarity network that controls numerous aspects of early development, including asymmetric cell division, asynchronous timing, and the asymmetric distribution of cell fate determinants. PAR-1 itself is asymmetrically distributed, with enrichment in the posterior cortex of the one-cell embryo as well as enrichment in P<sub>1</sub> relative to AB, whereas PAR-4 is evenly distributed (Rose and Gönczy, 2014). Work in human cells has shown that PAR-4 (STK11) phosphorylates and activates the kinase activity of PAR-1 (MARK2) (Lizcano et al., 2004), and thus it is possible to model a linear pathway whereby PAR-4 activates PAR-1 to limit the amount of CYB-3 that is inherited by P<sub>1</sub>. This type of pathway has been shown to control anterior enrichment of the MEX-5 RNA-binding protein (Griffin et al., 2011); however, MEX-5 asymmetry relies on differential diffusion of the protein within the cytoplasm, a mechanism that is unlikely to apply to CYB-3.

How then might CYB-3 asymmetry be achieved? One possibility is asymmetric distribution of the *cyb-3* mRNA; however, *in situ* hybridization data available in the Nematode Expression Database (NEXTDB; <http://nematode.lab.nig.ac.jp>) suggest that the *cyb-3* message is evenly distributed in two-cell embryos. Other possibilities include preferential mRNA translation in AB relative to P<sub>1</sub>, or enhanced CYB-3 proteolysis in P<sub>1</sub> relative to AB. I favor the second possibility, as existing data show that CYB proteins can be degraded in early embryos by pathways distinct from the canonical anaphase promoting complex (APC) pathway (Liu et al., 2004; Wang et al., 2013). These findings suggest the intriguing possibility that APC-independent pathways for CYB-3 proteolysis might function to promote the asymmetric distribution of CYB-3, and experiments are currently in progress to test this hypothesis.

Another interesting question raised by our work is whether other cyclins are also asymmetrically distributed in early embryos. Previous work has examined this for CYE-1 (cyclin E), and it is evenly distributed (Rivers et al., 2008); however, the intra-embryo distributions of the other CYBs, as well as of CYA-1 (cyclin A), have not been determined.

### CYB-3 and S-phase asynchrony

It is well established that cell division within the P-lineage is both asymmetric and asynchronous (Rose and Gönczy, 2014; Tavernier et al., 2015), and that the early cell divisions in the worm embryo are S/M cycles with no gap phases (Edgar and McGhee, 1988). For the two-cell embryo, the length of M-phase is similar between AB and P<sub>1</sub> (Brauchle et al., 2003), whereas the length of S-phase differs, and thus cell cycle asynchrony in the early embryo stems from a difference in the time required to complete DNA replication. How are the differential DNA replication dynamics for AB and P<sub>1</sub> established? Previous work has shown, in early S-phase, that replication is more robust in AB than in P<sub>1</sub> (Benkemoun et al., 2014), suggesting that replication initiates more efficiently in AB. More efficient initiation would allow AB to establish more replication forks in early S-phase, and this would in turn reduce replicon size and thereby allow faster completion of genome duplication. Previous work has also shown that PAR-1 and PAR-4 control replication in the early embryo, through an unknown mechanism, such that these factors repress replication in P<sub>1</sub>. In *par-1* and *par-4* mutants, replication in early S-phase is equivalent in two-cell embryos, and the blastomeres then enter mitosis in a synchronous manner (Benkemoun et al., 2014). I have shown here that CYB-3 is required for timely replication, that it is unevenly distributed in a manner dependent on *par-1* and *par-4*, and that it is functionally limiting in P<sub>1</sub> relative to AB. These findings, together with previous work (Benkemoun et al., 2014), suggest a simple model for S-phase asynchrony in which the asymmetric distribution of CYB-3 promotes more efficient replication in AB relative to P<sub>1</sub>, thereby allowing AB to complete S-phase before P<sub>1</sub> (Fig. 6D).

Although our data fit well with a differential replication model for cell cycle asynchrony, I note that previous work has identified an alternative possibility with the findings that the G2/M regulators CDC-25.1 and PLK-1 are asymmetrically distributed, in a *par*-dependent manner, in the early embryo (Budirahardja and Gönczy, 2008; Rivers et al., 2008). AB contains more CDC-25.1 and PLK-1 than does P<sub>1</sub>, and because these factors positively regulate mitotic entry it has been proposed that this imbalance allows AB to enter mitosis before P<sub>1</sub>. Indeed, partial depletion of these factors delays cell cycle progression, and P<sub>1</sub> is more delayed than AB (Budirahardja and Gönczy, 2008; Rivers et al., 2008), which is similar to what I have shown here for CYB-3. While it is clear that CDC-25.1/PLK-1 function is limiting in P<sub>1</sub>, it is not known how this would impact S-phase, and given that differential S-phase is the basis for cell cycle asynchrony it remains unclear if the CDC-25.1/PLK-1 asymmetry is itself important for asynchrony. I propose that the CDC-25.1/PLK-1 asymmetry evolved as a way to balance the CYB-3 asymmetry, so that equal ratios of CDK-1–CYB-3 to CDC-25.1/PLK-1 are present in both AB and P<sub>1</sub>. This would allow both cells to swiftly enter mitosis once replication has finished. In this scenario, an asymmetric distribution of CYB-3 allows differential DNA replication, and asymmetric distribution of CDC-25.1/PLK-1 ensures that mitotic entry is kinetically similar in AB and P<sub>1</sub>.

Although this model is consistent with all available data, further work is needed to unravel how CYB-3 is asymmetrically

distributed, and then to experimentally inactivate the asymmetric distribution without disturbing upstream polarity cues. Once this is accomplished, the role of CYB-3 asymmetric distribution in cell cycle asynchrony can be rigorously tested.

## MATERIALS AND METHODS

### C. elegans maintenance and RNAi

Worms were maintained according to standard procedures (Brenner, 1974). RNAi was performed by feeding (Timmons and Fire, 1998). Details of strains and RNAi conditions are provided in the supplementary Materials and Methods.

### Interphase timing of one-cell and two-cell embryos

Gravid adults were placed in a 5 µl drop of M9 on an 18 mm-square coverslip and sliced open with a 20-gauge needle to release embryos. Coverslips were then mounted on microscope slides containing a thin 1.8% agarose pad and embryos were imaged using an Olympus BX51 microscope equipped with DIC optics. Images shown in Fig. 1A, Fig. 2A and Fig. 5A were captured using the 40× objective and a Spot camera (Diagnostic Instruments).

### Immunostaining and microscopy

Embryos were permeabilized by freeze-cracking. Details of the antibodies used, including validation, as well as sample fixation, microscopy, image capture and image quantification are provided in the supplementary Materials and Methods.

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### Competing interests

The author declares no competing or financial interests.

### Author contributions

W.M.M. performed all experiments and wrote the manuscript.

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### Supplementary information

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