PLK-1 asymmetry contributes to asynchronous cell division of C. elegans embryos

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Acquisition of lineage-specific cell cycle duration is an important feature of metazoan development. In Caenorhabditis elegans, differences in cell cycle duration are already apparent in two-cell stage embryos, when the larger anterior blastomere AB divides before the smaller posterior blastomere P1. This time difference is under the control of anterior-posterior (A-P) polarity cues set by the PAR proteins. The mechanisms by which these cues regulate the cell cycle machinery differentially in AB and P1 are incompletely understood. Previous work established that retardation of P1 cell division is due in part to preferential activation of an ATL-1/CHK-1 dependent checkpoint in P1, but how the remaining time difference is controlled is not known. Here, we establish that differential timing relies also on a mechanism that promotes mitosis onset preferentially in AB. The polo-like kinase PLK-1, a positive regulator of mitotic entry, is distributed in an asymmetric manner in two-cell stage embryos, with more protein present in AB than in P1. We find that PLK-1 asymmetry is regulated by A-P polarity cues through preferential protein retention in the embryo anterior. Importantly, mild inactivation of plk-1 by RNAi delays entry into mitosis in P1, but not in AB, in a manner that is independent of ATL-1/CHK-1. Together, our findings support a model in which differential timing of mitotic entry in C. elegans embryos relies on two complementary mechanisms: ATL-1/CHK-1-dependent preferential retardation in P1 and PLK-1-dependent preferential promotion in AB, which together couple polarity cues and cell cycle progression during early development.

INTRODUCTION

Lineage specific cell cycle duration is crucial for metazoan development. For example, in Drosophila embryos, the expression pattern of the Cdc25 phosphatase string (stg) dictates the timing of the G2/M transition in different mitotic domains during the 14th cell cycle (Edgar and O’Farrell, 1990). stg mutant embryos arrest at the G2/M transition of cycle 14, whereas stg overexpression in interphase of cycle 14 leads to premature entry into mitosis and subsequent patterning defects (Edgar and O’Farrell, 1990). In Xenopus, both the Wee1 kinase and the Cdc25C phosphatase are essential for entry into mitosis and subsequent patterning defects (Edgar and O’Farrell, 1990). In C. elegans, a difference in cell cycle timing is apparent already in two-cell stage embryos, when the larger anterior blastomere AB divides ~2 minutes earlier than the smaller posterior blastomere P1. As in early embryos of many species, the cell cycle is rapid and alternates between S and M phases in early embryos of C. elegans. Thus, the ~2 minutes time difference correspond to ~15% of the entire duration of the AB cell cycle. The asynchrony between AB and P1 is entirely under the control of A-P polarity cues that are established by six PAR proteins and associated components, in concert with the actomyosin network (Aceto et al., 2006; Etemad-Moghadam et al., 1995; Guo and Kemphues, 1995; Guo and Kemphues, 1996; Levitan et al., 1994; Morton et al., 2002; Tabuse et al., 1998; Watts et al., 1996; Watts et al., 2000). When A-P polarity cues are defective, as for example in par-2 or par-3 mutant embryos, the first division is equal and the two resulting daughter cells enter mitosis in synchrony (Kemphues et al., 1988).

How A-P polarity cues control the asynchrony between AB and P1 is only partially understood. An aspect of the answer lies in an ATL-1/CHK-1-dependent checkpoint that is activated preferentially in P1, thus retarding entry into mitosis more in that blastomere and explaining ~40% of the time difference (Brauchle et al., 2003). Preferential checkpoint activation is largely abrogated in embryos that have normal A-P polarity, but which undergo an equal first cleavage following defective spindle positioning (Brauchle et al., 2003). These observations suggest that ATL-1/CHK-1 activation normally occurs preferentially in P1 because this smaller blastomere inherits less of a rate-limiting component for DNA replication following unequal first cleavage (Brauchle et al., 2003). These findings indicate also that there must be an ATL-1/CHK-1 independent and size-independent mechanism that regulates the remaining ~60% time difference. Compatible with the existence of a size-independent mechanism, microsurgery experiments established that AB enters mitosis earlier even if it becomes smaller than P1 because of the removal of cytoplasmic material (Schierenberg and Wood, 1985).

One attractive candidate for regulating the remaining time difference is the polo-like kinase PLK-1. First, Polo-like kinases 1 (Plk1) promote mitotic entry in several species (Abrieu et al., 1998; Lane and Nigg, 1996; Lenart et al., 2007; Sumara et al., 2004). Plk1 is thought to act at this cell cycle transition by phosphorylating the Myt1 kinase and the Cdc25 phosphatase. As a result, Myt1 is inactivated and Cdc25 is activated, which together promotes activation of Cdk1 and entry into mitosis (Inoue and Sagata, 2005; Kumagai and Dunphy, 1996; Qian et al., 2001; Roshak et al., 2000). In C. elegans, PLK-1 is also essential for M phase entry. Oocytes in plk-1(RNAi) animals fail to undergo nuclear envelope breakdown (NEDB) and do not complete the meiotic divisions (Chase et al., 2000). As anticipated, this phenotype is identical to that observed after RNAi-mediated inactivation of NCC-1, the Cdk1 homolog.

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acting during the meiotic and the mitotic divisions in early *C. elegans* development (Boxem et al., 1999). A second reason for considering PLK-1 as an attractive candidate for regulating the remaining time difference between AB and P1 is that examination of published data reporting the distribution of PLK-1 using N-terminal peptide antibodies (Chase et al., 2000) indicates that PLK-1 is more abundant in AB than in P1. Therefore, we set out to investigate whether PLK-1 plays a role in regulating the asynchrony between AB and P1 by promoting entry into mitosis preferentially in AB.

**MATERIALS AND METHODS**

**Nematode strains and RNA-mediated inactivation**

Transgenic worms expressing GFP-PAR-2 (Cuenca et al., 2003), GFP-PLK-1 (Leidel and Gönczy, 2003) or GFP-HIS-11 (Histone 2B) (Prattis et al., 2001) were grown at 24°C. Mutants of genotypes *par-3(i71) unc-32(e189)/qC1* (Kemphues et al., 1988), *par-1(it511) rol-4(sC8)/DnT1* (Gao and Kemphues, 1995) and *pie-1(zu154) unc-25(e156)/qC1* (Tenenhaus et al., 1998) were grown at 20°C, whereas temperature-sensitive *par-4(it57ks)* mutants (Morton et al., 1992) were grown at 15°C and shifted to 25°C for 24 hours before analysis.

The *ait-1(m853)* deletion allele was obtained from the National Bioresource Project, Japan. We balanced the mutation using nT11(GFP-MYO-2 unc-56) and genotyped the animals by PCR using *ait-1* specific primers. *ait-1(m853)* is probably a null allele because it harbors a 720 bp deletion that creates a frameshift and results in a premature stop codon.

**Indirect immunofluorescence**

Fixation and indirect immunofluorescence were performed essentially as described (Gönczy et al., 1999). The following primary mouse antibodies were used: α-tubulin (DM1A, Sigma; 1/200) and GFP (MAB3580, Chemicon; 1/200, to detect GFP-PAR-2). The following primary rabbit antibodies were used: PLK-1 C-terminal fusion protein (1/1500; this study), GFP (a gift from Viesutrn Simanis; 1/300, to detect GFP-PLK-1), NCC-1 (a gift from Andy Golden; 1/1000) and phospho-specific tyrosine 15 (pT15) antibody to detect NCC-1 phosphorylation (Calbiochem, 1/100). Secondary antibodies were goat anti-mouse coupled to Alexa 488 (1/500) and goat anti-rabbit coupled to Alexa 568 (1/1000) (Molecular Probes). Slides were counterstained with ~1 μg/ml Hoechst 33258 (Sigma) to reveal DNA. Images were taken on a LSM510 Zeiss confocal microscope. For quantification of the ratio of PLK-1 in AB versus P1, images were taken in the linear intensity range with a 63× lens on a Zeiss Axioplan 2 microscope equipped with a 12-bit Diagnostic Instrument Spot RT cooled CCD camera and analyzed using MetaMorph software. For pT15 staining, 20 confocal sections encompassing the AB and P1 nuclei were taken every 0.45 μm with a 63× objective and the maximum intensity projection was constructed using ImageJ.

**FRAP experiments and image analysis**

FRAP was performed on an LSM510 Meta Zeiss confocal microscope, acquiring one image every 10 seconds at each time point using a 63× lens, with zoom 2, average 2 and unidirectional scanning of 512×512 pixels at 9% laser power, resulting in acquisition times of ~800 mseconds. For photobleaching, the whole anterior or posterior half of a metaphase one-cell stage GFP-PLK-1 embryo was bleached with 30 iterations at 100% laser power. The data were analyzed using ImageJ by measuring the average intensity of the anterior or posterior cytoplasmic area, excluding centrosomes and kinetochores, whose frequent movements could generate measurement errors. The intensity in the embryo anterior in the five images before photobleaching was averaged and assigned a value of 1, whereas the intensity in the bleached area just after photobleaching was assigned a value of 0.

**Time-lapse microscopy**

Embryos were analyzed by time-lapse DIC microscopy at ~23°C as described (Gönczy et al., 1999). The timing of cytokinesis in P0 (measured at the time of cleavage furrow initiation) as well as that of NEBD in AB and P1 (measured at the time of nuclear membrane disappearance) were determined. The time separating cytokinesis in P0 from NEBD in either AB or P1 corresponds to interphase.

Embryos expressing GFP-Histone 2B were imaged using dual time-lapse fluorescent and DIC microscopy as described (Brauchle et al., 2003), and the timing of the metaphase to anaphase transition determined in P0, AB and P1.

**Quantification of PLK-1 levels**

PLK-1 levels were quantified in fixed wild-type, par-2(RNAi) and par-3(i71) embryos. To obtain reliable values, we normalized cytoplasmic PLK-1 levels with respect to centrosomal PLK-1 levels in the same embryo. Moreover, the analysis was conducted in metaphase and early anaphase one-cell stage embryos because the centrosomal signal is strong and reproducible at these stages, and because anterior and posterior levels can be quantified simultaneously in the same cell. As PLK-1 is distributed symmetrically in par-2(RNAi) and par-3(i71) embryos, PLK-1 levels in the anterior and the posterior were averaged in these cases. Embryos which were partially stained or subjected to incomplete RNAi-mediated inactivation of par-2 were not considered.

**RESULTS**

**PLK-1 is distributed asymmetrically in early *C. elegans* embryos**

To investigate whether the asymmetry in PLK-1 distribution that is apparent in a published report (Chase et al., 2000) reflects bona fide PLK-1 distribution, and not merely that of a particular epitope, we raised and affinity-purified polyclonal antibodies against a C-terminal fragment of the protein (Fig. 1A). These antibodies recognize a single band at the expected size of ~70 kDa in wild-type embryonic extracts by western blot analysis (Fig. 1B, lane 1). This
band is significantly reduced in plk-1(RNAi) embryonic extracts, demonstrating specificity (Fig. 1B, lane 2). We used these antibodies to examine PLK-1 distribution in early C. elegans embryos. As shown in Fig. 1C, we found that PLK-1 is distributed in a uniform manner in the cytoplasm just after meiotic exit. PLK-1 distribution becomes asymmetric shortly thereafter, with more protein present on the anterior side of the embryo (Fig. 1D). This asymmetry persists throughout the one-cell stage and until the end of the two-cell stage (Fig. 1E,F; data not shown). Line scans quantifying pixel intensities confirm these observations (Fig. 1C-F). These antibodies also label centrosomes (Fig. 1E, arrowheads), the midbody (Fig. 1E,F, arrows) and kinetochores (data not shown), as reported previously (Chase et al., 2000). The signals detected by these antibodies in wild-type embryos are absent from embryos depleted of PLK-1 by RNAi (Fig. 1G), demonstrating specificity. We found an analogous distribution using antibodies directed against GFP in transgenic animals expressing GFP-PLK-1, although the asymmetry is somewhat less pronounced in this case (Fig. 1H).

Overall, these observations lead us to conclude that PLK-1 distribution is asymmetric in one- and two-cell stage C. elegans embryos, with more protein being present on the anterior side.

Achieving PLK-1 asymmetry

We next set out to investigate how PLK-1 asymmetry is achieved. We first addressed whether the underlying mechanism is active solely in the one-cell stage and results in asymmetry at the two-cell stage merely because the first cleavage separates the anterior and posterior pools of PLK-1. Alternatively, the mechanism at the root of PLK-1 asymmetry could be active also during the second cell cycle. To distinguish between these possibilities, we examined PLK-1 asymmetry at the second cell cycle in zen-4(RNAi) embryos, which
are defective in cytokinesis (Raich et al., 1998). As shown in Fig. 2B, PLK-1 asymmetry is maintained despite cytokinesis failure (B). Whereas microtubules are not essential for PLK-1 asymmetry as the asymmetry is maintained and even slightly increased upon α-tubulin depletion (C), the actomyosin network is needed (D). (E,F) Wild-type (E) and partial plk-1(RNAi) (F) embryos stained for PLK-1 (shown alone in the left panels and in red in the merged panels), α-tubulin (green) and DNA (blue). Overall PLK-1 levels are diminished in partial plk-1(RNAi) embryos (F, top panel). To better illustrate the alteration in ratio between AB and P1, a second image of the same partial plk-1(RNAi) embryo is shown (F, bottom panel), in which intensities were increased so as to match those in AB in the wild-type embryo. (G) Average ratios, along with s.e.m., of PLK-1 in AB versus P1 in wild-type and partial plk-1(RNAi) embryos stained as in E and F. Wild type, 1.52±0.05, n=64; partial plk-1(RNAi), 1.97±0.13, n=16. Student’s t-test, P=2.6×10⁻¹¹. The star indicates that the difference with wild type is statistically significant.

We then tested whether the microtubule cytoskeleton or the actomyosin network is required for PLK-1 asymmetry. To test the role of microtubules, we depleted α-tubulin using tba-2(RNAi) (Sonneville and Gönczy, 2004). We found that PLK-1 asymmetry is still apparent and even slightly accentuated in tba-2(RNAi) embryos (Fig. 2C), indicating that microtubules are dispensable for PLK-1 asymmetry. To examine the role of the actomyosin network, we depleted the non-muscle myosin II NMY-2 (Guo and Kemphues, 1995). As shown in Fig. 2D, PLK-1 asymmetry is abolished in nmy-2(RNAi) embryos, indicating that the actomyosin network is required, directly or indirectly, for PLK-1 asymmetry.

A fraction of PLK-1 is retained at the embryo anterior

Next, we considered whether PLK-1 distribution is asymmetric because a fraction of the protein is retained in the embryo anterior. To test this possibility, we designed a Fluorescence Recovery After Photobleaching (FRAP) experiment to probe the mobility of the posterior and anterior pools of PLK-1. To probe the mobility of GFP-PLK-1 located in the embryo posterior, we photobleached the anterior half of the embryo and assayed fluorescence intensity over time in both posterior and anterior halves (Fig. 3A,B). We found that the posterior pool of GFP-PLK-1 moves readily into the anterior half, such that fluorescence intensity in the two halves equilibrates ~90 seconds after photobleaching (Fig. 3B). Conversely, to probe the mobility of GFP-PLK-1 located into the anterior, we photobleached the posterior half of the embryo and similarly assayed fluorescence intensity (Fig. 3C,D). Importantly, we found in this case that even though the bulk of the anterior pool of GFP-PLK-1 moves readily into the posterior half, a fraction of GFP-PLK-1 is retained in the embryo anterior, such that fluorescence intensity in the two halves remains different at 90 seconds after photobleaching and thereafter (Fig. 3D).

As PLK-1 can be retained in the embryo anterior, we reasoned that the extent of asymmetry may increase when overall PLK-1 levels are diminished, because the remaining protein would be preferentially retained in the anterior, leaving less protein left for the posterior. To test this possibility, we compared the ratio of PLK-1 in AB versus P1 in the wild type with that observed after partial RNAi-
mediated inactivation of PLK-1. We found this ratio to be 1.52±0.05 in the wild-type (Fig. 2E,G) and 1.97±0.13 in embryos partially depleted of PLK-1 (Fig. 2F,G). Although more complex mechanisms can be envisaged, this increase in ratio, together with the outcome of the FRAP experiment, is compatible with a model in which PLK-1 asymmetry is achieved through protein retention in the embryo anterior.

**PLK-1 asymmetry is controlled by A-P polarity cues**

As the time difference between AB and P1 is entirely under the control of A-P polarity cues (Kemphues et al., 1988), if PLK-1 were to play a role in controlling this time difference, then PLK-1 asymmetry should be regulated by polarity cues. To address this possibility, we first examined when PLK-1 becomes asymmetric with respect to establishment of A-P polarity. Using GFP-PAR-2 as a polarity marker, we found that PLK-1 distribution becomes asymmetric shortly after polarity establishment (Fig. 4A,B), compatible with PLK-1 asymmetry being regulated by A-P polarity cues. To test whether this is the case, we examined PLK-1 distribution in embryos with impaired PAR-1, PAR-2, PAR-3, PAR-4 or PAR-5 function. Importantly, we found that PLK-1 distribution is symmetric in such embryos (Fig. 4D-H). Similarly, PLK-1 distribution is symmetric in pck-3(RNAi) and mex-5/6(RNAi) embryos, which also have defective A-P polarity (Cuenca et al., 2003; Tabuse et al., 1998) (data not shown). Therefore, we conclude that PLK-1 asymmetry is regulated by A-P polarity cues.

We then investigated whether polarity cues regulate PLK-1 asymmetry separately from asymmetric spindle positioning and cell fate determination, both of which are also controlled by A-P polarity (reviewed by Gönczy and Rose, 2005). To this end, we examined goa-1/gpa-16(RNAi) embryos, in which the first division is equal as a result of symmetric spindle positioning (Colombo et al., 2003; Gotta and Ahringer, 2001), as well as pie-1(zu154) mutant embryos, in which the fate of the P1 blastomere is altered (Tenenhaus et al., 1998; Tenenhaus et al., 2001). We found that PLK-1 distribution is asymmetric in such embryos (Fig. 4I,J), indicating that A-P polarity cues regulate PLK-1 asymmetry separately from spindle positioning and cell fate determination.

**PLK-1 levels correlate with the timing of mitotic entry upon PAR-2 and PAR-3 inactivation**

We set out to test whether PLK-1 levels are important for dictating the timing of mitosis onset. We reasoned that an analysis of PLK-1 levels and mitotic timing in embryos depleted of PAR-2 and PAR-3 may shed light on this issue. In the absence of PAR-2, both blastomeres at the two-cell stage resemble AB in terms of spindle orientation and cortical marker distribution (Boyd et al., 1996; Kemphues et al., 1988). Conversely, in the absence of PAR-3, both blastomeres resemble P1 in these respects (Etemad-Moghadam et al., 1995; Kemphues et al., 1988). Accordingly, we found that the timing of mitotic entry of both blastomeres in par-2(RNAi) embryos resembles most that of AB in the wild type, whereas the timing of both blastomeres in par-3(it71) mutant embryos is similar to that of P1 in the wild-type (Fig. 5A-F; see Table S1 and Movies 1-4 in the supplementary material). Quantification of fixed specimens established that PLK-1 levels in par-2(RNAi) embryos are similar to those in the anterior of wild-type embryos, whereas those in par-3(it71) embryos are similar to those in the posterior of wild-type embryos (Fig. 5G). We conclude that levels of PLK-1 correlate with the timing of mitotic entry upon depletion of PAR-2 and PAR-3.
PLK-1 asymmetry contributes to asynchronous entry into mitosis in AB and P1
We next set out to address whether the asymmetric distribution of PLK-1 is important for asynchronous division of AB and P1. Strong RNAi-mediated inactivation of plk-1 prevents completion of the meiotic divisions (Chase et al., 2000) and conditional mutant alleles are not available, precluding an analysis in two-cell stage embryos using strong reduction of function conditions. Thus, we performed partial plk-1(RNAi) and analyzed cell cycle progression using time-lapse DIC microscopy. We found that mitotic entry in partial plk-1(RNAi) embryos is delayed somewhat in AB, but more extensively in P1, resulting in a significant increase in the ratio of the duration of interphase in P1 over the duration of interphase in AB (RI) (see Tables S1 and S2 in the supplementary material). Although this observation indicates that PLK-1 levels are crucial for timely entry into mitosis, chromosomes are often mis-segregated in these partial plk-1(RNAi) embryos, which in theory could contribute to altering cell cycle progression. Therefore, we conducted yet milder RNAi-mediated inactivation of plk-1, such that chromosome segregation defects are not observed. Importantly, we found that whereas the timing of cell cycle progression in AB is not affected in these mild plk-1(RNAi) embryos, entry into mitosis is delayed in P1 (Fig. 6A-B; see Table S1, Movies 5 and 6 in the supplementary material). As a result, RI is increased compared with the wild type (Fig. 6E; see Table S2 in the supplementary material). To corroborate these observations, we analyzed the timing of events in embryos expressing GFP-Histone 2B to monitor precisely the metaphase to anaphase transition. We found again that whereas cell cycle duration is not perturbed in AB in mild plk-1(RNAi) embryos, a delay is observed in P1 (see Fig. S1A,B, and Movies 11 and 12 in the supplementary material), resulting in an increased ratio of cell cycle duration in P1 over that in AB (see Fig. S1E and Table S3 in the supplementary material). As P1 has lower levels of PLK-1 than AB in the wild type, it is presumably more sensitive to PLK-1 diminution, accounting for the observed increased asynchrony upon mild RNAi-mediated depletion. Taken together, these results suggest that PLK-1 levels are important for timing mitotic entry and that PLK-1 asymmetry contributes to the asynchronous division of AB and P1.

As PLK-1 promotes mitotic entry by ultimately activating the Cdk1 NCC-1, we anticipated that interfering with NCC-1 function may have consequences on differential cell cycle duration resembling those observed in mild plk-1(RNAi) embryos. NCC-1 is distributed symmetrically in AB and P1 (see Fig. S2A-C in the supplementary material) (Boxem et al., 1999), but is activated first in AB, as evidenced by the fact that phospho-specific antibodies that recognize the inactive form of NCC-1 disappear first from AB (see Fig. S2D-F in the supplementary material) (Hachet et al., 2007). As anticipated, we found that entry into mitosis in mild ncc-1(RNAi) embryos is delayed to a larger extent in P1 than in AB (Fig. 6C; see Table S1 and Movie 7 in the supplementary material), resulting in an increased RI compared with the wild type (Fig. 6E; see Table S2 in the supplementary material).
in the supplementary material), reminiscent of the increase observed in mild $plk-1(RNAi)$ embryos. Even though in principle factors other than PLK-1 could be responsible for differential activation of NCC-1, this phenotypic similarity is compatible with the notion that PLK-1 asymmetry results in differential NCC-1 activation.

To address whether such a differential impact on mitotic entry in AB and P1 is a general feature of slowing cell cycle progression, we analyzed embryos depleted of ATP-2, a β subunit of the catalytic moiety of ATP synthase (Tsang et al., 2001). Although cell cycle progression is considerably slower overall than in the wild-type, we found that mitotic entry is delayed to the same extent in AB and P1 in $atp-2(RNAi)$ embryos (Fig. 6D; see Table S1 and Movie 8 in the supplementary material), such that RI is indistinguishable from the wild type (Fig. 6E; see Table S2 in the supplementary material). Taken together, these results indicate that mild depletion of PLK-1 or of the downstream Cdk1 kinase NCC-1, but not any delay in cell cycle progression, retards entry into mitosis preferentially in P1.

**Fig. 5.** PLK-1 levels correlate with the timing of mitotic entry after $par-2$ or $par-3$ inactivation. (A,B,D,E) Images from DIC time-lapse microscopy of wild-type (A,D), $par-2(RNAi)$ (B) or $par-3(it71)$ embryos (E) (see corresponding Movies 1–4). Black arrowheads indicate cleavage furrow ingression; white arrowheads with intact outline indicate intact nuclei; white arrowheads with broken outline indicate nuclei undergoing NEBD, which is apparent by loss of the smooth line corresponding to the nuclear envelope; circles indicate centrosomes in AB, the separation of which gives an indication of the advancement of mitosis. Cleavage furrow ingression at the onset of cytokinesis in P0 is defined as $t=0$, and the time after that is indicated in seconds. (C,F) Average duration of interphase ±s.e.m. in AB and P1 in wild-type and $par-2(RNAi)$ (C) or wild-type and $par-3(it71)$ (F) embryos. See Table S1 in the supplementary material for numerical values and statistical analysis. (G) Relative PLK-1 levels ±s.e.m. in anterior and posterior of one-cell stage wild-type, $par-2(RNAi)$ and $par-3(it71)$ embryos. Wild-type anterior, 0.68±0.04, $n=12$; wild-type posterior, 0.45±0.03, $n=12$; $par-2(RNAi)$, 0.61±0.08, $n=9$; $par-3(it71)$, 0.47±0.06, $n=7$. Student’s t-test for wild-type anterior and $par-2(RNAi)$, $P=0.1$; for wild-type posterior and $par-2(RNAi)$, $P=1.7×10^{-4}$; for wild-type anterior and $par-3(it71)$, $P=7.36×10^{-7}$; for wild-type posterior and $par-3(it71)$, $P=0.42$. PLK-1 levels in $par-2(RNAi)$ embryos are similar to those of the wild-type anterior, whereas PLK-1 levels in $par-3(it71)$ are similar to those of the wild-type posterior.
**PLK-1-mediated differential timing is independent of ATL-1**

As differential timing in AB and P1 is known to rely in part on preferential retardation of mitotic entry in P1 through activation of an ATL-1/CHK-1 dependent checkpoint (Brauchle et al., 2003), we investigated whether the impact of PLK-1 depends on this checkpoint.

First, we addressed whether ATL-1/CHK-1 regulates PLK-1 asymmetric distribution. We examined the ratio of PLK-1 in AB versus P1 in *atl-1(tm853)* embryos, which are checkpoint defective and thus have decreased RI (Fig. 7F,H; see Table S2 and Movie 9 in the supplementary material) (Brauchle et al., 2003), as well as in *div-1(RNAi)* embryos, which engage this checkpoint more than in the wild type owing to defective DNA replication and thus exhibit increased RI (Brauchle et al., 2003; Encalada et al., 2000). If ATL-1/CHK-1 regulates PLK-1 asymmetry, then the ratio of PLK-1 in AB versus P1 should decrease in *atl-1(tm853)* embryos and increase in *div-1(RNAi)* embryos. We found instead that PLK-1 asymmetry is indistinguishable from the wild type in both cases (Fig. 7A–D), demonstrating that alterations in checkpoint signaling do not affect PLK-1 asymmetry.

To address unambiguously whether PLK-1 functions independently of ATL-1/CHK-1, we compared *atl-1(tm853)* embryos with *atl-1(tm853)* embryos treated with mild *plk-1(RNAi)*. If the impact of PLK-1 on cell cycle progression requires ATL-1/CHK-1 function, then mild *plk-1(RNAi) should not alter the timing of events observed in *atl-1(tm853)* embryos. In contrast to this prediction, however, we found that mild *plk-1(RNAi)* treatment of *atl-1(tm853)* embryos retards entry into mitosis in P1 and thus increases RI compared with *atl-1(tm853)* mutant embryos (Fig. 7G,H; see Table S2 and Movie 10 in the supplementary material), as expected if the impact of PLK-1 is independent of ATL-1/CHK-1. To corroborate these observations, we conducted analogous experiments in embryos expressing GFP-Histone 2B. As reported in Fig. 7I–E in the supplementary material, these experiments similarly established that mild *plk-1(RNAi)* treatment of *atl-1(tm853)* mutant embryos retards entry into mitosis in P1 and thus increases RI compared with *atl-1(tm853)* mutant embryos (see Table S3, and Movies 13 and 14 in the supplementary material).

Taken together, these results establish that the impact of PLK-1 asymmetry on differential timing of mitotic entry in AB and in P1 does not invoke ATL-1/CHK-1.

**DISCUSSION**

How cell cycle progression is modulated during development remains incompletely understood. Here, we demonstrate that asymmetric distribution of the cell cycle regulator PLK-1 couples A-P polarity cues to the timing of mitosis in two-cell stage *C. elegans* embryos.

**Mechanisms underlying PLK-1 asymmetry**

How is PLK-1 asymmetry established? In principle, asymmetry could result from *plk-1* mRNA being enriched at the embryo anterior. This does not appear to be the case, however, because mRNA localization is typically mediated through sequences in the 3’ UTR (Evans et al., 1994; Ogura et al., 2003), which are absent from the *gfp-plk-1* construct that nevertheless results in anterior enrichment (Leidel and Gönczy, 2003). Accordingly, in situ hybridization indicates that *plk-1* mRNA is distributed uniformly in two-cell stage embryos (http://nematode.lab.nig.ac.jp/db2/ShowGeneInfo.php?celk=CELK02520). Therefore, PLK-1 asymmetry is regulated at the protein level. Although other mechanisms can be envisaged, including preferential degradation in the embryo posterior, our findings taken together support a model in which PLK-1 asymmetry is established through preferential PLK-1 protein retention in the embryo anterior.

It will be interesting to investigate the molecular underpinning of PLK-1 anterior retention. Three putative PKC-3 phosphorylation sites are present in PLK-1 (Y.B. and P.G., unpublished) and perhaps phosphorylation of PLK-1 by anteriorly localized PKC-3 contributes to PLK-1 asymmetry. Another possibility is suggested by the observation that PLK-1 distribution is uniform in *nmy-2(RNAi)* embryos. Although this may be an indirect consequence from the polarity defects known to arise following NMY-2 depletion (Cuenca et al., 2003; Guo and Kemphues, 1996), the requirement for NMY-2 in PLK-1 localization could be more direct. NMY-2 is enriched on the anterior cortex of one- and two-cell stage embryos (Munro et al., 2004), and may help enrich PLK-1 in the vicinity of...
a structure or complex that promotes anterior retention. In Drosophila, the non-muscle myosin II Zipper is needed for asymmetric localization of Miranda to the basal side of neuroblasts (Barros et al., 2003).

**PLK-1 levels dictate timing of mitotic entry and contribute to asynchronous division in two-cell stage C. elegans embryos**

Previous work established that PLK-1 is essential for M-phase entry in C. elegans, as oocytes from animals severely depleted of PLK-1 do not undergo NEDB or complete the meiotic divisions (Chase et al., 2000). Here, we demonstrate in addition that levels of PLK-1 are important for dictating the timing of mitotic entry. Indeed, partial depletion of PLK-1 delays mitotic entry in AB and more so in P1, whereas milder depletion of PLK-1 delays mitotic entry merely in P1.

Where in the cell are the substrates that are phosphorylated by PLK-1 to promote entry into mitosis? Such substrates may reside in the cytoplasm, where PLK-1 enrichment is most apparent. Alternatively, as centrosomes are important for dictating the timing of mitosis in C. elegans (Hachet et al., 2007), and as PLK-1 is present at centrosomes, perhaps it is centromeral PLK-1 that is relevant for mitotic timing. Because centromeral PLK-1 readily exchanges with PLK-1 in the cytoplasm (Leidel and Gönczy, 2003), an excess of cytoplasmic PLK-1 in the anterior may enhance PLK-1 loading at centrosomes in AB compared with P1. Accordingly, levels of centromeral PLK-1 increase during the cell cycle and are high in AB earlier than in P1 (Y.B. and P.G., unpublished).

**Coupling polarity cues and cell cycle progression**

Our findings taken together support a model in which two mechanisms together ensure coupling between A-P polarity cues and cell cycle progression in two-cell stage C. elegans embryos (Fig. 7I). The first mechanism entails preferential activation of ATL-1/CHK-1 in the P1 blastomere and contributes to asynchrony by retarding entry into mitosis in P1 more than in AB (Brauchle et al., 2003). Such preferential checkpoint activation may result from the unequal first cleavage, as preferential activation is largely abrogated in embryos that divide symmetrically. Thus, A-P polarity cues regulate this first mechanism by ensuring asymmetric spindle positioning and unequal cleavage. These earlier findings predicted the existence of another mechanism, also regulated by A-P polarity cues, but independent of size difference and of ATL-1/CHK-1.

**Fig. 7. PLK-1 mediated differential timing is independent of ATL-1.**

A-C Wild-type (A), atl-1(tm853) (B) or div-1(RNAi) (C) two-cell stage embryo stained for PLK-1 (shown alone in left panels and in red in the merged panels), α-tubulin (green) and DNA (blue). PLK-1 distribution is asymmetric as in the wild type. D Average ratios, along with s.e.m., of PLK-1 in AB versus P1 determined on fixed wild-type, atl-1(tm853) and div-1(RNAi) embryos. Wild type, 1.52±0.05, n=64; atl-1(tm853), 1.48±0.12, n=9, P=0.58, compared with the wild type (two-tailed Student’s t-test); div-1(RNAi), 1.45±0.17, n=8, P=0.32, compared with the wild type (two-tailed Student’s t-test).

E-G Images from DIC time-lapse microscopy of wild-type (E), atl-1(tm853) (F) or atl-1(tm853) embryos treated with mild plk-1(RNAi) (G) (see corresponding Movies 5, 9-10 in the supplementary material). See legend of Fig. 5 for explanation of symbols and timing.

H Average ratios ±s.e.m. of the duration of interphase in P1 over the duration of interphase in AB [(IP1)/(IAB)] in embryos of the indicated genotypes. The star denotes that the difference with wild type is statistically significant. See Table S2 in the supplementary material for numerical values and statistical analysis. I Working model. Preferential promotion of mitotic entry in AB through the presence of more PLK-1, together with preferential retardation of mitotic entry in P1 through engagement of an ATL-1/CHK-1-dependent checkpoint, ensure differential cell cycle duration in two-cell stage C. elegans embryos.
Our present work indicates that this second mechanism entails PLK-1 asymmetry, which promotes entry into mitosis in AB earlier than in P1. The evidence supporting such a mechanism can be summarized as follows. First, PLK-1 is distributed asymmetrically, with more protein present in AB than in P1. Second, PLK-1 asymmetry is regulated by A-P polarity cues, independently of cell size, as PLK-1 is still asymmetric in goa-1parison-16(RNAi) embryos. Third, the time difference between AB and P1 increases when PLK-1 is depleted, and this occurs in an ATL-1/CHK-1 independent manner. Interestingly, Polo is also asymmetrically distributed in the germlarium of Drosophila, where it promotes entry into meiosis and ooocyte determination (Mirouse et al., 2006). Furthermore, in Drosophila neuroblasts, Polo phosphorylates Pdc and thus ensures efficient segregation of the fate determinant Numb during asymmetric cell division (Wang et al., 2007). Together with our own work, these findings indicate that the evolutionarily conserved Polo-like kinas es may broadly serve to couple cell cycle timing and developmental decisions.

**Note added in proof**

While this manuscript was under review, another study reported that PLK-1 also regulates MEX-5/6 function (Nishi et al., 2008).

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**Supplementary material**

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/135/7/1303/DC1

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**References**


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**Development**

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