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The CeCDC-14 phosphatase is required for cytokinesis in the *Caenorhabditis elegans* embryo

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In all eukaryotic organisms, the physical separation of two nascent cells must be coordinated with chromosome segregation and mitotic exit. In *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* this coordination depends on a number of genes that cooperate in intricate regulatory pathways termed mitotic exit network and septum initiation network, respectively. Here we have explored the function of potentially homologous genes in a metazoan organism, *Caenorhabditis elegans*, using RNA-mediated interference. Of all the genes tested, only depletion of CeCDC-14, the *C. elegans* homologue of the budding yeast dual-specificity phosphatase Cdc14p (Clp1/Flp1p in fission yeast), caused embryonic lethality. We show that CeCDC-14 is required for cytokinesis but may be dispensable for progression of the early embryonic cell cycles. In response to depletion of CeCDC-14, embryos fail to establish a central spindle, and several proteins normally found at this structure are mislocalized. CeCDC-14 itself localizes to the central spindle in anaphase and to the midbody in telophase. It colocalizes with the mitotic kinesin ZEN-4, and the two proteins depend on each other for correct localization. These findings identify the CDC14 phosphatase as an important regulator of central spindle formation and cytokinesis in a metazoan organism.

Introduction

The correct segregation of genetic material is paramount for the growth and development of all eukaryotic organisms. Of particular importance, cells must spatially and temporally coordinate spindle positioning and chromosome segregation with the physical separation of the nascent cells. In recent years, substantial progress has been made towards understanding this coordination in both budding yeast and fission yeast. In *Saccharomyces cerevisiae*, exit from mitosis is coupled to correct spindle positioning by a signalling cascade termed the mitotic exit network (MEN)* (Jaspersen et al., 1998). The MEN senses the position of the spindle pole bodies (SPBs) in reference to mother and daughter cells, and delays Cdk1 (Cdc28p) inactivation until one SPB has entered the bud (Bardin et al., 2000; Pereira et al., 2000). The position of the SPB determines the nucleotide state of an SPB-associated small GTPase, Tem1p (Shirayama et al., 1994; Bardin et al., 2000; Pereira et al., 2000). This protein in turn controls a cascade of serine/threonine kinases, comprising Cdc15p (Schweitzer and Philippsen, 1991) and Dbf2p (Johnston et al., 1990; Mah et al., 2001), which acts upon the most downstream target of the MEN, the protein phosphatase Cdc14p (Shou et al., 1999; Visintin et al., 1999). Whereas Cdc14p is sequestered and kept inactive in the nucleolus from early mitosis to anaphase, activation of the mitotic exit network results in its release from the nucleolus. Cdc14p then dephosphorylates and functionally activates several Cdk1 substrates, including Hct1/Cdh1, an accessory factor of the anaphase promoting complex/cyclosome (APC/C), and the Cdk1 inhibitor Sic1p (Jaspersen et al., 1999; Shou et al., 1999; Visintin et al., 1999). As a result, Cdk1 activity drops and exit from mitosis ensues.

In *Schizosaccharomyces pombe*, gene products apparently homologous to the components of the budding yeast MEN have originally been identified as regulators of septation, a process akin to cytokinesis (for review see Balasubramanian et al., 2000; Bardin and Amon, 2001). Hence, the corresponding regulatory pathway is referred to as the septum initiation network (SIN). Although many SIN genes are structurally similar to MEN genes, they appear to regulate primarily the deposition of the septum rather than the inactivation of Cdk1 (Cdc2p) (for review see Le Goff et al., 1999; McCollum and Gould, 2001). As a result, SIN mutants continue to seg-

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*Abbreviations used in this paper: APC/C, anaphase promoting complex/cyclosome; DIC, differential interference contrast; MEN, mitotic exit network; pNPP, para-nitrophenyl phosphate; RNAi, RNA-mediated interference; SIN, septum initiation network; SPB, spindle pole body.

Key words: CDC14; cytokinesis; central spindle; ZEN-4; AIR-2

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regate their DNA without undergoing cytokinesis. In a cytokinesis mutant, the SIN is also required to keep Cdk1 activity low and prevent entry into the next cell cycle. This latter function appears to be mediated by Clp1p/Flp1p, the *S. pombe* homologue of the Cdc14p phosphatase. Specifically, it appears that Clp1p/Flp1p may dephosphorylate both Cdc25p andWee1p, thereby maintaining Cdk1 in an inactive, tyrosine-phosphorylated state (Trautman et al., 2001). Fission yeast Clp1p/Flp1p also seems to be regulated by sequestration to the nucleolus during interphase. However, during mitosis, it has been observed on the SPBs, the spindle microtubules, and the cell division septum (Cueille et al., 2001; Trautman et al., 2001).

In metazoan organisms, the coordination of late mitotic events and cytokinesis with chromosome segregation remains poorly understood. Considering that metazoan cell division displays several features that are not encountered in yeast and vice-versa, the mechanisms regulating the exit from mitosis and the onset of cytokinesis in animal cells are expected to differ in important aspects from those emerging in yeast. For instance, considering that the nuclear envelope breaks down during mitosis in most multicellular eukaryotes, it is by no means clear that the nucleolar sequestration mechanism that controls Cdc14 activity in yeast is also operating in metazoan species.

In animal cells, the site of cleavage furrow formation is determined by the position of the mitotic spindle (Cao and Wang, 1996). During anaphase, a structure of antiparallel microtubule bundles, the so-called central spindle, forms between the separating chromosomes. This central spindle is essential for the completion of cytokinesis, as mutations in several of its components cause a failure in cytokinesis (Williams et al., 1995; Adams et al., 1998; Raich et al., 1998; Jantsch-Plunger et al., 2000). How the central spindle is assembled has not yet been elucidated in detail, but data from several organisms attribute an important role in the bundling of midzone microtubules to a mitotic kinesin-related motor protein, termed CHO1/Mklp1 in mammals (Sellitto and Kuriyama, 1988; Nislow et al., 1992), pavarotti in *Drosophila* (Adams et al., 1998), and ZEN-4 in *C. elegans* (Powers et al., 1998; Raich et al., 1998). Interestingly, the formation of the central spindle is prevented if Cdk1 activity persists, even though chromosome separation is not affected (Murray et al., 1996; Wheatley et al., 1997) Therefore, the reversal of the Cdk1-mediated inhibition of central spindle formation may constitute a key element in the coordination of cytokinesis with chromosome segregation.

Inspection of metazoan genomes reveals putative homologues for several components of the yeast MEN/SIN pathways, but for others, candidate homologues cannot readily be identified. Thus, it remains to be determined to what extent the yeast MEN/SIN circuits have been conserved during evolution. This question is particularly pertinent, as significant differences in the wiring of these circuits have been described even between budding yeast and fission yeast (for review see Balasubramanian et al., 2000; Bardin and Amon, 2001; McCollum and Gould, 2001). Of all the MEN/SIN gene products identified so far, the most highly conserved component is the phosphatase Cdc14p (Clp1p/Flp1p). In human cells, two CDC14 isoforms, CDC14A and CDC14B, have been described (Li et al., 1997, 2000). The function of these proteins remains poorly understood, although a recent study suggests a role for mammalian CDC14A in the centrosome cycle (Mailand et al., 2002). In support of this view, CDC14A localizes to centrosomes, whereas overexpressed CDC14B has been found at the nucleolus (Mailand et al., 2002). Furthermore, like its budding yeast counterpart, CDC14A can dephosphorylate Hct1/Cdh1p, at least in vitro (Bembenek and Yu, 2001).

To determine which, if any, of the identified yeast MEN/SIN components could play a role in mitotic exit and/or cytokinesis in metazoan cells, we studied the embryo of the nematode *Caenorhabditis elegans*, a widely used model for metazoan cell division. Specifically, we used RNA-mediated interference (RNAi) to deplete *C. elegans* worms of potential MEN/SIN homologues, and scored embryos for cell cycle–related phenotypes. Of all candidate MEN/SIN components analyzed here, only the depletion of CeCDC-14, the *C. elegans* homologue of the *S. cerevisiae* Cdc14p phosphatase, produced embryonic lethality. Careful analysis of the corresponding phenotype revealed that CeCDC-14 is dispensable for progression of the nuclear cycles in the early embryo, but essential for central spindle formation and cytokinesis. This suggests that central spindle formation in animal cells depends critically on the dephosphorylation of one or several Cdk1-substrates.

Results

RNAi analysis of putative *C. elegans* homologues of yeast MEN/SIN proteins

A search of the *C. elegans* genome for potential homologues of the yeast MEN/SIN genes revealed several candidate genes, although for some of these genes the sequence similarities were only limited and for *TEM1*, *CDC15*, and *BFA1* no putative homologues could be identified (Table I). All candidate genes were tested in *C. elegans* for a possible function in a hypothetical MEN/SIN-like regulatory network, using RNAi to deplete the corresponding products. For depletion of putative MEN/SIN gene products, both RNAi feeding and injection methods (Montgomery and Fire, 1998; Timmons et al., 2001) were tried to maximize the probability of functional inactivation. The results of these experiments are summarized in Table I. A priori, we had expected that depletion of a gene product required for the regulation of mitotic exit or the onset of cytokinesis should result in embryonic lethality. However, of all components tested, only the depletion of the *C. elegans* homologue of the budding yeast Cdc14p phosphatase caused embryonic lethality in the offspring of injected worms (Table I). The corresponding phenotype will be described in more detail below. Depletion of two other gene products also caused specific, albeit different, phenotypes. In the case of the ORF T19A5.2, a potential homologue of the fission yeast kinase Sid1p, a striking degeneration of the germline in both RNAi-injected and -fed worms was observed. In injected worms the production of oocytes ceased, and instead the germ line appeared to cellularize. Depletion of the ORF T20F10.1, one of the putative Dbf2p homologues, caused the offspring of injected worms to arrest at an early larval

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Table I. **Putative *C. elegans* homologues of MEN/SIN genes**[a]

S. cerevisiae	*S. pombe*		Phenotype	
MEN	**SIN**	**Putative *C. elegans* homologue**	**Feeding**	**Injection**
TEM1	*spg1*$^+$	?		
BFA1	*byr4*$^+$	?		
BUB2	*cdc16*$^+$	C33F10.2	no phenotype	no phenotype
CDC15	*cdc7*$^+$	?		
DBF2/DBF20	*sid2*$^+$	T20F10.1	no phenotype	F_1 arrest as L1[b]
		R11G1.4	no phenotype	no phenotype
MOB1/MOB2	*mob1*$^+$	T12B3.4	no phenotype	no phenotype
		F38H4.10	no phenotype	no phenotype
		F09A5.4	no phenotype	no phenotype
?	*sid1*$^+$	T19A5.2	F_1 sterile with disorganized germline	Germline in injected worms degenerates Production of embryos ceases F_1 are sterile[b]
CDC14	*flp1*$^+$/*clp1*$^+$	C17G10.4	no phenotype	embryonic lethality[b]

[a]Summary of RNAi phenotypes observed after depletion of putative *C. elegans* homologues of yeast MEN/SIN genes.
[b]For illustration, see Fig. S1 (available at http://www.jcb.org/cgi/content/full/jcb.200202054/DC1).

stage (L1). These results could suggest that *C. elegans* protein kinases related to yeast Sid1p and Dbf2p may be involved in cell cycle regulation, in particular tissues and developmental stages of the worm. However, given the absence of a striking phenotype in the early *C. elegans* embryo, the study of these kinases was not pursued. Instead, our subsequent analyses were focused on the *C. elegans* homologue of the budding yeast Cdc14p phosphatase, the ostensibly best conserved component of the yeast MEN/SIN regulatory circuits.

CeCDC-14 is a phosphatase and localizes to the central spindle and the midbody

Our analysis of the *C. elegans* genome had revealed only one ORF with significant homology to *CDC14,* C17G10.4, but because WormBase (http://www.wormbase.org) predicted three potential splice variants of this ORF, total worm cDNA preparations were subjected to RT-PCR. This analysis revealed a single product, corresponding to the smallest splice variant, C7G10.4c. Furthermore, sequencing of several ESTs derived from C17G10.4 also yielded only sequences corresponding to C17G10.4c (unpublished data). Similarly, only a single product, corresponding to the molecular mass predicted for C17G10.4c (76.7 kD) could be detected at the protein level (see below). Thus, although it would be premature to exclude the existence of minor splice variants, we conclude that the 76.7-kD product of C17G10.4c represents the major CDC14 phosphatase in *C. elegans*.

To demonstrate that this ORF encodes a functional phosphatase, CeCDC-14 was expressed as a His-tagged fusion protein in bacteria. The purified protein was then used in phosphatase assays with the artificial substrate para-nitrophenyl phosphate (pNPP). 6His–CeCDC-14 hydrolyzed pNPP with a specific activity of 96 nmoles phosphate released min^{-1} mg^{-1}, close to the specific activity reported for Cdc14p of *S. cerevisiae* (Taylor et al., 1997). This phosphatase activity was completely abolished upon mutation of the presumptive catalytic cysteine 295 to serine. Furthermore, dephosphorylation of pNPP by CeCDC-14 was efficiently inhibited by 200 μM sodium-orthovanadate (5% activity of control reaction) and only weakly inhibited by 20 mM sodium fluoride or 100 nM okadaic acid (72 and 87% activity of control reactions, respectively). A similar sensitivity to inhibitors had also been reported for Cdc14p (Taylor et al., 1997). Taken together, these data strongly indicate that the protein encoded by ORF C17G10.4c is the *C. elegans* homologue of *S. cerevisiae* Cdc14p. Thus, we hereafter refer to this protein as CeCDC-14.

To facilitate the study of CeCDC-14 in the embryonic cell cycles of *C. elegans*, a polyclonal antibody was raised against a COOH-terminal fragment of CeCDC-14 (spanning amino acids 352–605). This fragment encompasses a region of the phosphatase domain that would be predicted to be conserved in all potential splice variants discussed above. Yet, in Western blots on total worm extracts, the affinity-purified antibody recognized a single band migrating at ca. 76 kD, very close to the size predicted for the smallest splice-variant (Fig. 1 A, lane 1). Furthermore, recombinant untagged CeCDC-14 comigrated exactly with the band detected in total worm extract (Fig. 1 A, lane 2), and both signals were completely eliminated upon addition of purified His-tagged CeCDC-14 (Fig. 1 A, lanes 3 and 4). Together, these results demonstrate the specificity of our antibody, and they confirm the identification of the endogenous protein as CeCDC-14.

The localization of CeCDC-14 was analyzed in wild-type *C. elegans* embryos, using the affinity-purified anti–CeCDC-14 antibodies for immunofluorescence microscopy. During interphase and early mitosis, anti–CeCDC-14 antibodies produced only weak diffuse staining, but no labeling of specific cellular structures (unpublished data). However, in anaphase, striking staining of the central spindle area was observed in anaphase (Fig. 1 B, top, arrowhead). Later in mitosis, during telophase, this staining compacted to a single dot that was positioned between the two daughter cells, highly reminiscent of the midbody (Fig. 1 B, bottom, arrowhead). Older embryos displayed multiple dots in the cytoplasm or between cells, again consistent with midbody staining (not depicted; see Fig. 7, bottom, left). Attesting to the specificity of this staining, neither the preimmune serum nor the affinity-purified serum preincubated with recombinant CeCDC-14 produced any distinct label-

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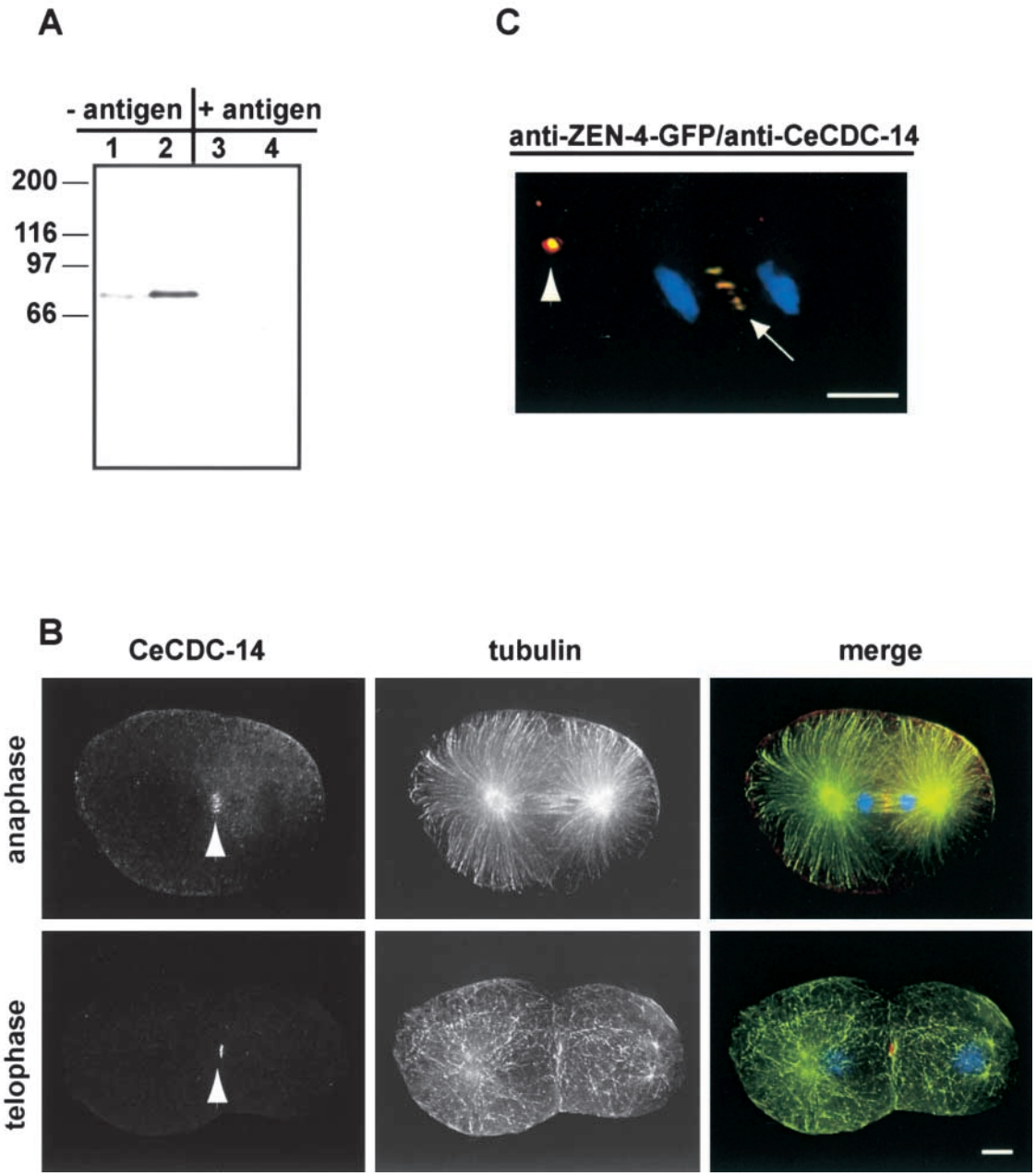

Figure 1. **CeCDC-14 localizes to the central spindle in anaphase and the midbody in telophase.** (A) Total worm extract (lanes 1 and 3) and lysate of insect cells infected with recombinant baculovirus encoding CeCDC-14 (lanes 2 and 4) were probed by immunoblotting with affinity-purified anti–CeCDC-14 antibodies, in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of purified recombinant 6His–CeCDC-14. (B) Wild-type *C. elegans* embryos were subjected to immunofluorescence microscopy using affinity-purified anti–CeCDC-14 antibodies. Tubulin staining is shown in green, anti–CeCDC-14 staining in red, and DAPI in blue. Arrowheads mark the central spindle (top) and the midbody (bottom panel). (C) ZEN-4–GFP (MG170) embryos in the first zygotic division were stained with goat anti-GFP (green) and rabbit-anti–CeCDC-14 (red) antibodies. Yellow color indicates colocalization. The polar body extrusion remnant is indicated by an arrowhead and the central spindle by an arrow. Bars, 5 μm.

ing (unpublished data). In contrast to recent reports on Cdc14 localizations in other organisms (Shou et al., 1999; Visintin et al., 1999; Bembenek and Yu, 2001; Mailand et al., 2002; Pereira et al., 2002), no staining of centrosomes or nuclei could be seen (Fig. 1 B).

The staining patterns produced by anti–CeCDC-14 antibodies were very reminiscent of those observed with antibodies directed against the central spindle components ZEN-4, a mitotic kinesin (Powers et al., 1998; Raich et al., 1998), and its partner protein CYK-4, a Rho-GAP (Jantsch-Plunger et al., 2000; Mishima et al., 2002). To directly explore a possible colocalization between CeCDC-14 and ZEN-4, worms expressing a functional ZEN-4–GFP fusion protein (Kaitna et al., 2000) were stained with antibodies against GFP and CeCDC-14. As illustrated in Fig. 1 C, the two antigens displayed extensive colocalization at both the central spindle and the polar body (arrow and arrowhead, respectively).

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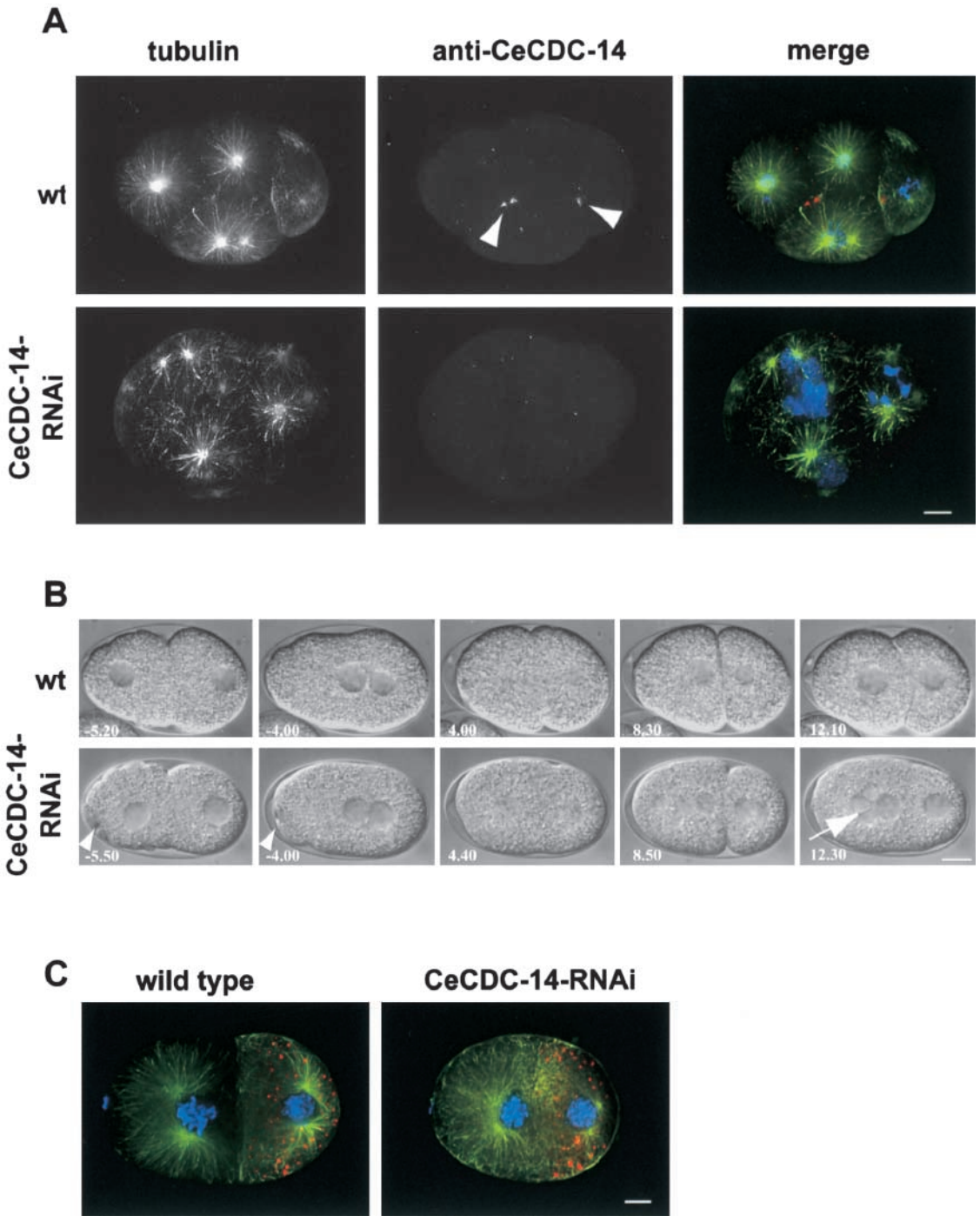

Figure 2. **Depletion of CeCDC-14 leads to failure of cytokinesis in *C. elegans* embryos.** (A) Wild-type and CeCDC-14–depleted embryos at equivalent stages were stained with anti-tubulin (green) and anti–CeCDC-14 (red) antibodies. DAPI staining is shown in blue. Arrows point to midbody remnants. Bar, 5 μm. (B) Wild-type and CeCDC-14–depleted embryos were filmed using differential interference contrast optics. Time is indicated in minutes at the bottom of each frame in reference to nuclear envelope break down (0.0). Note that the CeCDC-14–depleted embryo failed polar body extrusion (bottom panel −5.50 min and −4.00 min, arrowheads). The extra DNA masses became visible as additional nuclei after nuclear envelope reformation (11.50 min, arrow). The cleavage furrow ingressed almost to wild-type levels but then regressed rapidly (8.50 and 12.30 min). Note the extruded polar bodies in the wild-type embryo (12.10 min). Bar, ~10 μm. (C) Wild-type and CeCDC-14–depleted embryos in telophase were stained with antibodies against PGL-1, a P granule component (red). Tubulin is stained in green and DAPI is in blue. Bar, 5 μm.

RNA-mediated interference with CeCDC-14 leads to failure of cytokinesis

The localization of CeCDC-14 to the central spindle and the midbody suggested an involvement of this protein in cytokinesis. To directly test this possibility, worms were injected with dsRNA corresponding to CeCDC-14. 24 h later, worms were dissected and the corresponding embryos analyzed. Most strikingly, all embryos derived from

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Table II. **Time course of CeCDC-14 RNAi**

Hours past injection	Embryonic lethality	First division cytokinesis defect	Polar body extrusion fails	Length of mitosis[a]
0–12	25.6% (92/358)	ND	ND	ND
12–18	62.8% (235/374)[b]	ND	ND	ND
18–24	91.1% (194/213)	7/7	6/7	14 min, 13 s ± 1 min, 24 s (n = 6)
24–30	100% (220/220)	12/13	11/13	16 min, 2 s 1 min, 49 s (n = 6)
30–36	100% (170/170)	16/16	12/16	17 min, 54 s ± 2 min, 42 s (n = 5)
Wild-type	0% (0/200)	0/10	0/10	14 min, 24 s ± 1 min, 1 s (n = 5)

[a]The length of mitosis was measured as the time from the meeting of the pronuclei to the reformation of both nuclear envelopes after completed/failed cytokinesis.
[b]Some of the worms that hatched shortly before the lethal period displayed disorganized germlines or died as larvae.

CeCDC-14 RNAi-treated worms were multinucleate, suggesting that they had undergone multiple cytokinesis failures. Identical results were obtained, regardless of the timing of dissection after worm injection (unpublished data; Table II). Analysis of CeCDC-14–depleted embryos by immunofluorescence microscopy with anti–CeCDC-14 antibodies (n = 25) did not show any staining, confirming the efficacy of protein elimination by RNAi (Fig. 2 A).

To examine the nature of the observed cytokinesis defect in more detail, embryos from worms injected with CeCDC-14–dsRNA were filmed using differential interference contrast (DIC) optics. As depicted in Fig. 2 B, such embryos

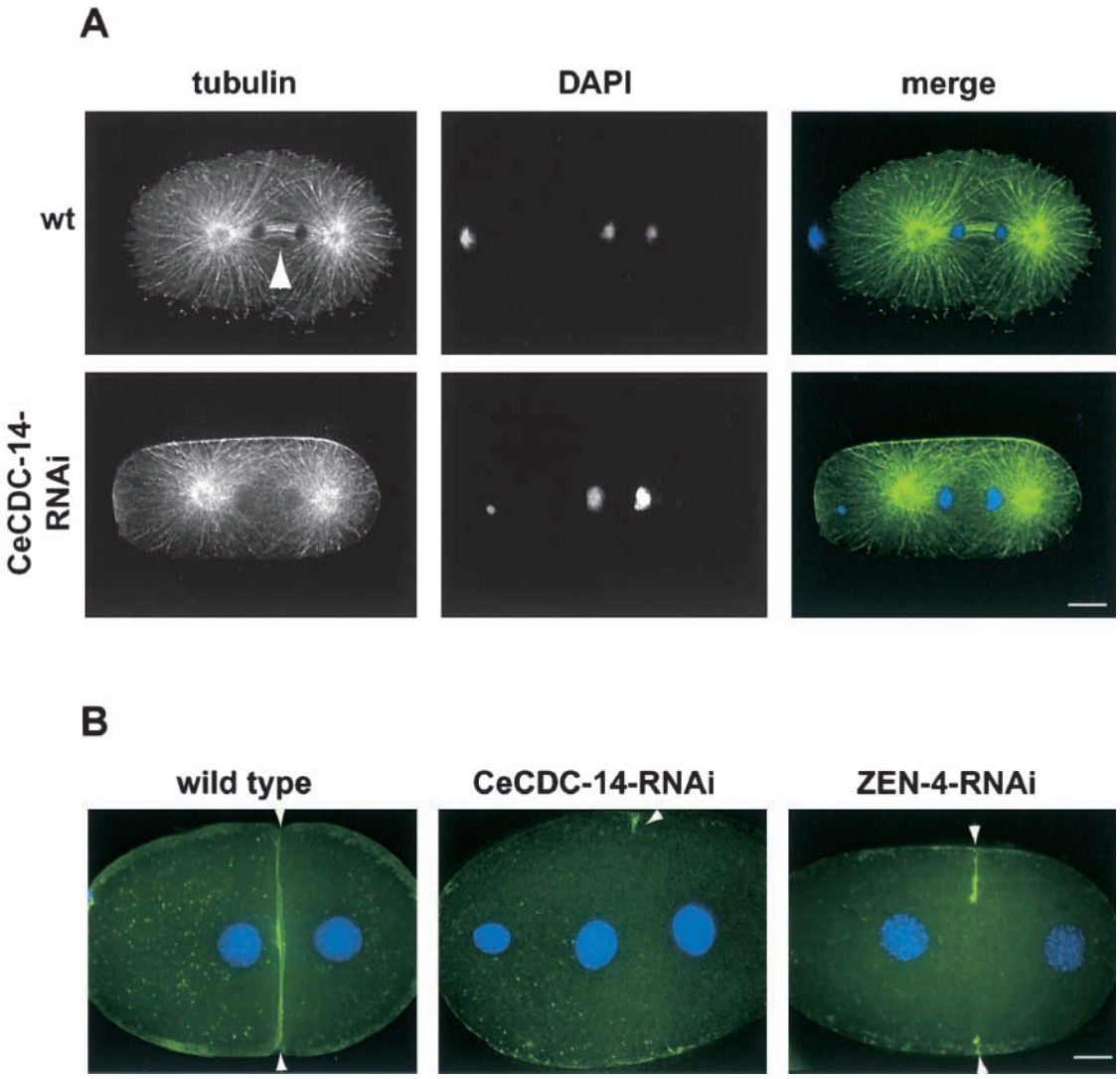

Figure 3. **CeCDC-14–depleted embryos lack bundled midzone microtubules.** (A) Wild-type and CeCDC-14–depleted embryos in the first anaphase were stained with anti-tubulin antibodies (green). DAPI staining is shown in blue. Arrowheads point to the central spindle (top), which is absent in CeCDC-14–depleted embryos. (B) Wild-type, CeCDC-14– and ZEN-4–depleted embryos in telophase were fixed with paraformaldehyde and stained with FITC-phalloidin. All three embryos showed cortical actin staining but only partial actin rings at the cleavage furrow were observed in CeCDC-14– and ZEN-4–depleted embryos (arrowheads). Bar, 5μm.

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progressed normally into the first mitosis and most of them also initiated cleavage furrow ingression in anaphase. In fact, regardless of the time of analysis after injection, most of the embryos (30/36) exhibited a deep cleavage furrow; however, subsequently, the furrows regressed and cytokinesis invariably failed. This phenotype is remarkably similar to the one observed in ZEN-4–depleted embryos (Powers et al., 1998; Raich et al., 1998). In addition to this cytokinesis failure in telophase, most of the embryos (29/36) also failed polar body extrusion in meiosis (Fig. 2 B, arrowhead, bottom, −5.50 and −4.00 min), leading to a more than 2N-DNA content in the zygotic embryo and multiple nuclei at later stages (Fig. 2 B, bottom, arrow, 12.30 min). These results indicate that the main defect caused by the depletion of CeCDC-14 is a pronounced failure of cytokinesis.

In a minor proportion of embryos (6/36), the furrow only pinched in marginally before it regressed and cytokinesis failed. In some of these embryos, the pseudocleavage also appeared weaker or the spindle was placed more centrally than observed in wild-type embryos. Similar features have been observed in mutants defective in the establishment of polarity (Shelton et al., 1999; Rappleye et al., 2002) raising the possibility that the absence of CeCDC-14 might also have caused a polarity defect. However, when CeCDC-14–depleted embryos were stained with antibodies against P granules, these were found to display the same posterior localization as in wild-type embryos (Fig. 2 C). Staining with antibodies against PAR-2 and PAR-3, two further markers of polarity, also failed to reveal aberrations in the establishment of polarity, and most CeCDC-14–depleted embryos also displayed the characteristic flattening of the posterior spindle pole in anaphase (unpublished data; Keating and White, 1998). Thus, our results fail to support a major, direct role of CeCDC-14 in the establishment of polarity. On the other hand, considering that ~15% of CeCDC-14–depleted embryos did give indications of a polarity defect, it would be premature to rigorously exclude such a role. It remains possible that very low levels of residual phosphatase activity could suffice for a polarity-related function.

The central spindle fails to form in worms injected with CeCDC-14 dsRNA

Problems with cytokinesis could either reflect a failure to establish a stable central spindle (Jantsch-Plunger et al., 2000; Powers et al., 1998; Raich et al., 1998), or a failure of the actomyosin ring to assemble and contract correctly (Swan et al., 1998; Shelton et al., 1999). In order to inspect the state of the central spindle and the actin cytoskeleton in CeCDC-14–depleted embryos, microtubules were stained with anti-tubulin antibodies and F-actin with phalloidin. In embryos injected with CeCDC-14–dsRNA, bundled microtubules characteristic of the central spindle were absent, whereas the astral microtubules appeared normal (25 out of 25 embryos) (Fig. 3 A). Analysis of F-actin using FITC-phalloidin (Fig. 3 B) revealed the expected cortical actin staining in wild-type ($n = 15$), as well as in CeCDC-14– ($n = 7$) and ZEN-4–depleted embryos ($n = 9$). The depleted embryos showed only partial actomyosin rings in telophase (Fig. 3 B), but this result needs to be interpreted with caution. As shown above (Fig. 2 B), the furrows in these depleted embryos regressed very rapidly, making it extremely difficult to capture the instant of maximal ingression for immunocytochemical analysis. Thus, we do not believe that either CeCDC-14 or ZEN-4 play a direct role in actomyosin ring formation, in agreement with previous reports on ZEN-4 (Powers et al., 1998; Severson et al., 2000). Instead, the regression of the contractile ring observed in CeCDC-14–depleted embryos most likely stems from a failure of the central spindle to form correctly. Thus, our data identify CeCDC-14 as a novel regulator of central spindle formation in metazoan cells.

Both ZEN-4 and AIR-2 are mislocalized in CeCDC-14–depleted embryos

The bundling of the central spindle microtubules in the *C. elegans* embryo has previously been attributed to the action of the ZEN-4/CYK-4 complex (Powers et al., 1998; Raich et al., 1998; Jantsch-Plunger et al., 2000). A homologous complex called centralspindlin has also been identified in human cells, and has been shown to induce microtubule bundling in vitro (Mishima et al., 2002). In view of these findings, the lack of bundled microtubules in the spindle midzone of CeCDC-14–depleted embryos could be explained if the ZEN-4/CYK-4 complex failed to localize correctly. To examine this possibility, the localization of ZEN-4 was examined in both wild-type and CeCDC-14–depleted embryos (Fig. 4 A). In wild-type embryos, ZEN-4 was detected at the spindle midzone and the midbody, confirming previous results (Powers et al., 1998; Raich et al., 1998). In contrast, CeCDC-14–depleted embryos showed virtually no staining for ZEN-4 in either anaphase or telophase ($n = 20$).

Another component required for normal cytokinesis in metazoan cells is the chromosomal passenger kinase AIR-2 (Aurora B) (for review see Adams et al., 2001a). In wild-type *C. elegans* embryos, AIR-2 localizes to the kinetochores of chromosomes in early mitosis, and to the central spindle and midbody in anaphase and telophase (Schumacher et al., 1998). In ZEN-4–depleted embryos, which lack dense microtubule bundles between the chromosome masses, AIR-2 localization is restricted to small microtubule bundles (Powers et al., 1998). Similarly, we observed that some microtubules were still labeled by anti–AIR-2 antibodies in CeCDC-14–depleted embryos ($n = 5$), even though no compact central spindles were assembled (Fig. 4 B, bottom). This suggests that the transfer of AIR-2 from kinetochores to microtubules is not impaired by the absence of CeCDC-14.

Failure of ZEN-4–GFP to localize to the spindle midzone in CeCDC-14–RNAi embryos

From the analysis of fixed CeCDC-14–depleted embryos, it was impossible to determine whether the mislocalization of ZEN-4 was due to an inability of ZEN-4 to localize to the central spindle, or alternatively, reflected an unstable association of ZEN-4 with the spindle midzone. The latter mechanism had previously been described to underlie the ZEN-4 mislocalization phenotype in embryos injected with dsRNA for the *C. elegans* INCENP homologue, ICP-1 (Kaitna et al., 2000). To distinguish between the two possibilities, worms

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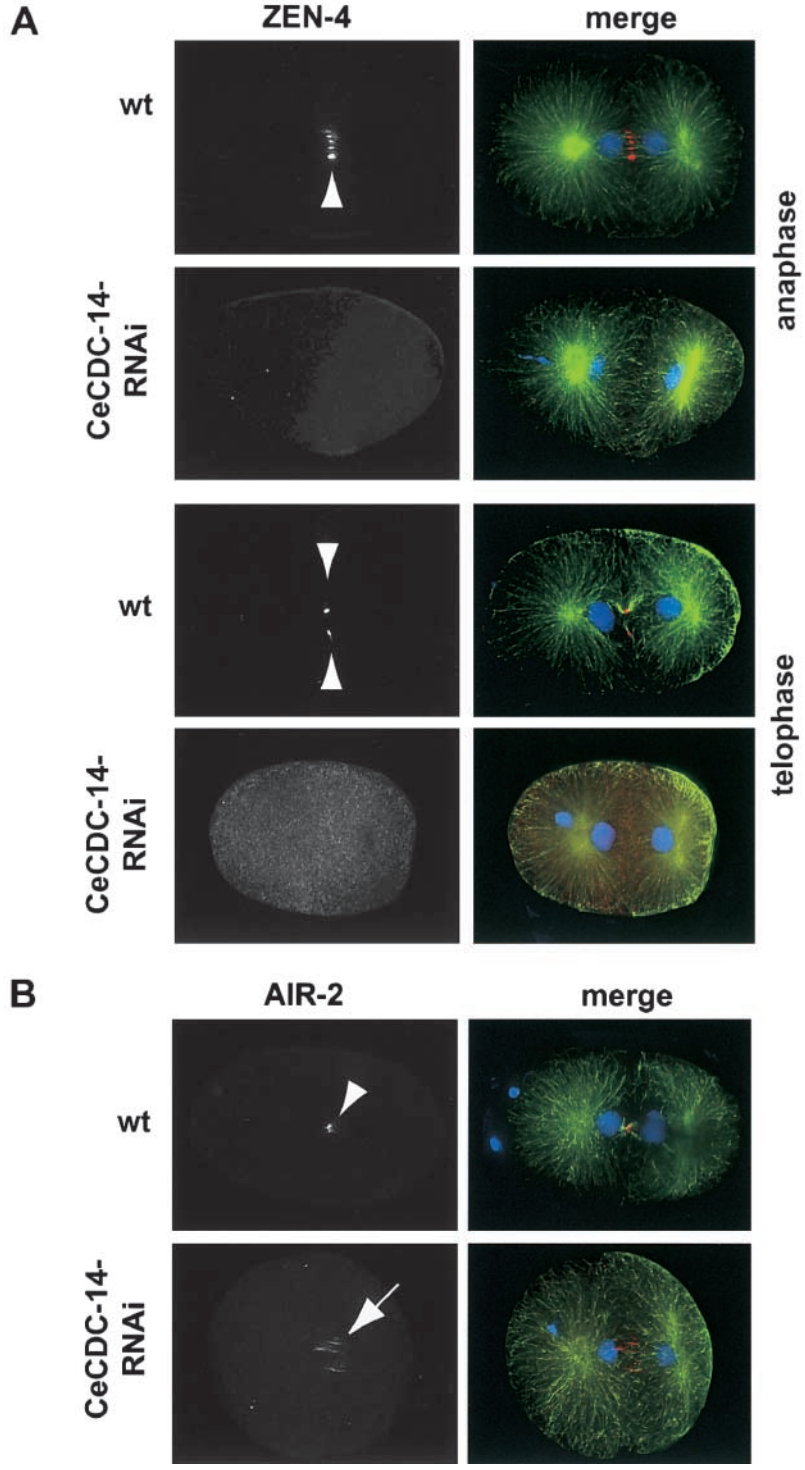

Figure 4. **ZEN-4 and AIR-2 are mislocalized in CeCDC-14–depleted embryos.** (A) Wild-type and CeCDC-14–depleted embryos were stained with anti-tubulin (green) and anti–ZEN-4 antibodies (red). (B) Wild-type embryos and embryos depleted of CeCDC-14 by RNAi were stained with antibodies recognizing AIR-2 (red). Note that in the CeCDC-14–depleted embryo some microtubules were stained with anti–AIR-2 antibody (bottom row, arrow) whereas the chromosomes were not recognized by the antibody. DNA staining is in blue. Arrowheads point to the central spindle and midbodies.

carrying a functional ZEN-4–GFP fusion (Kaitna et al., 2000) were injected with CeCDC-14 dsRNA, and CeCDC-14–depleted and control embryos were then observed in real time. In wild-type embryos, ZEN-4–GFP began to localize to the central spindle in anaphase, and subsequently formed a bright spot at the position of the midbody in telophase ($n = 4$) (Fig. 5, left). However, in embryos depleted of CeCDC-14, no transient accumulation of ZEN-4–GFP could be observed at any time point ($n = 6$) (Fig. 5, right two panels). From the comparison of these results with those reported for embryos depleted of ICP-1 (Kaitna et al., 2000), it seems clear that depletion of CeCDC-14 disturbs the organization of the central spindle more completely than depletion of ICP-1.

CeCDC-14 and ZEN-4 are interdependent in their localization

To characterize the relationship between CeCDC-14 and ZEN-4 in more detail, embryos were also depleted of ZEN-4 by RNAi, and then analyzed by immunofluorescence mi-

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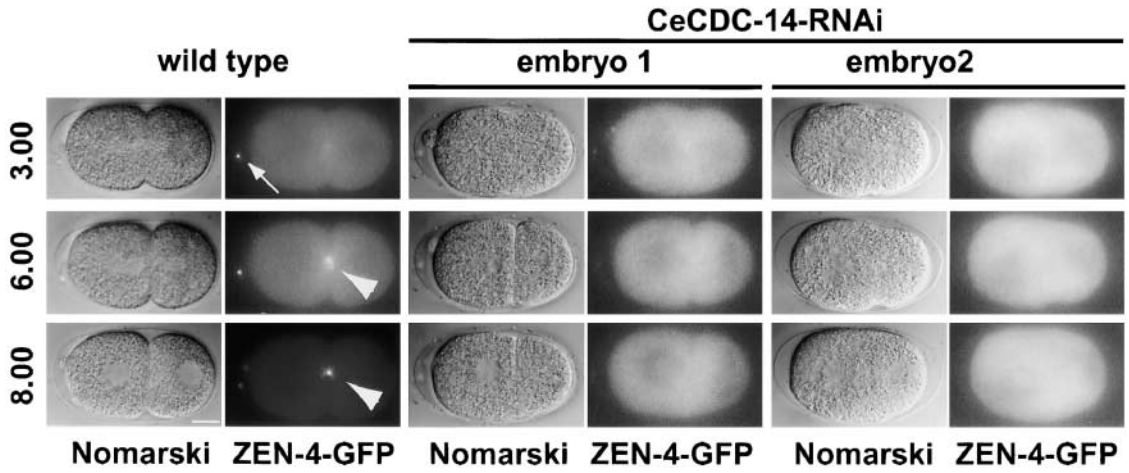

Figure 5. **ZEN-4-GFP fails to localize in vivo to the midzone of CeCDC-14–depleted embryos.** Untreated (left two columns) and CeCDC-14–RNAi-injected (right four columns) ZEN-4–GFP (MG170) embryos were observed throughout the first division. The time indicated is in reference to nuclear envelope break down (0.0 min). Two CeCDC-14–depleted embryos are shown. Note the dot-like ZEN-4–GFP signal in the wild-type embryo at the site of polar body extrusion (arrow) that was absent in the CeCDC-14–depleted embryos. Similarly, ZEN-4–GFP accumulated at the central spindle in anaphase (6 min, arrowhead) and at the midbody during cytokinesis (8 min, arrowhead) in wild-type but not in CeCDC-14–depleted embryos. Bar, ~10 μm.

croscopy, using anti–CeCDC-14 antibodies. As shown in Fig. 6, CeCDC-14 was completely lost from both the central spindle and the midbody in ZEN-4–depleted embryos ($n = 25$) (Fig. 6). The same result was obtained in CYK-4–depleted embryos (unpublished data). Together with the data presented in Fig. 4, this indicates that CeCDC-14 and the ZEN-4/CYK-4 complex are mutually dependent on each other for localization. In parallel, we also examined embryos depleted for AIR-2. In addition to a severe cytokinesis defect, such AIR-2-depleted embryos display problems with DNA segregation during anaphase, leading to chromatin bridges and unsegregated DNA masses in telophase (Schu-

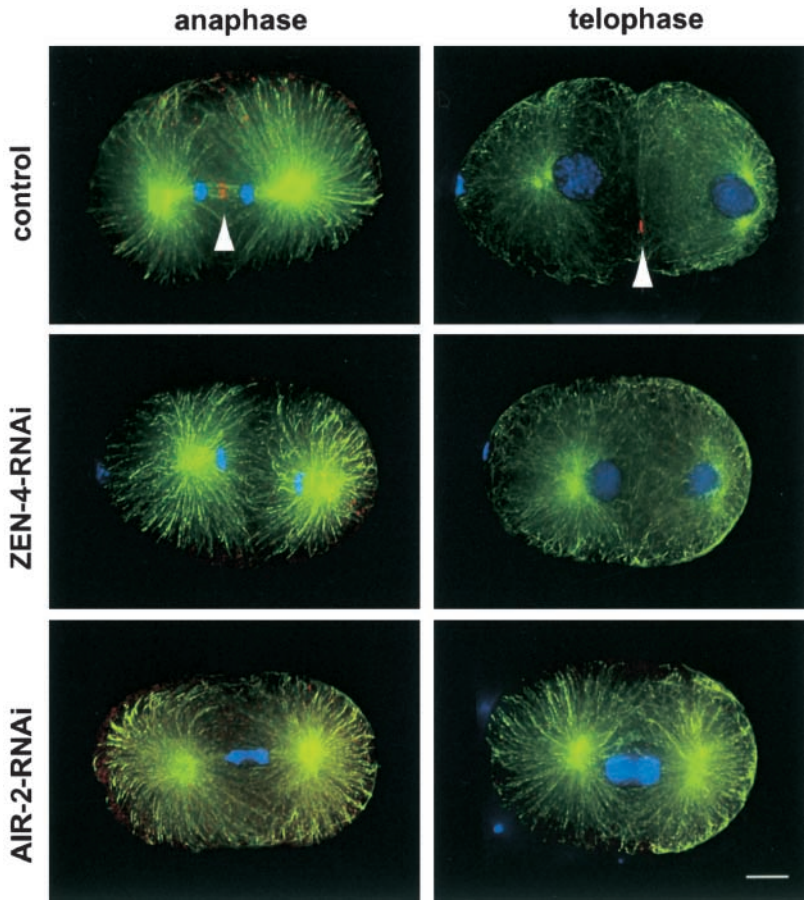

Figure 6. **ZEN-4–depleted embryos do not show localized CeCDC-14 staining.** Control (UNC-22-RNAi), ZEN-4– and AIR-2–depleted embryos were stained with antibodies against CeCDC-14 (red) and α-tubulin antibodies (green). DAPI staining is in blue. Arrowheads point to central spindle and midbody. Bar, 5 μm.

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Table III. **Cell cycle analysis using histone H2B-GFP**

	Wild-type[a]	CeCDC-14-RNAi[a,b]
1st to 2nd mitotic metaphase	17 min, 22 s ± 1 min, 45 s	18 min, 13 s ± 2 min, 1 s
Nuclear envelope breakdown to 1st metaphase	3 min, 6 s ± 19 s	3 min, 7 s ± 24 s
1st mitotic metaphase to chromosome decondensation	2 min, 25 s ± 11 s	2 min, 32 s ± 22 s

[a]$n = 7$ for both wild-type and CeCDC-14–depleted embryos.
[b]For this experiment CeCDC-14 depleted embryos were used 30–36 h postinjection in order to ensure maximal depletion of the protein.

macher et al., 1998; Kaitna et al., 2000). In most AIR-2–depleted embryos examined with anti-CeCDC-14 antibodies, no localized CeCDC-14 staining could be seen ($n = 20$) (Fig. 6, bottom).

Depletion of CeCDC-14 does not affect cell cycle progression in *C. elegans* embryos

As described above, embryos depleted of CeCDC-14 clearly failed cell division, but remarkably they appeared to continue in the cell cycle. In fact, CeCDC-14–depleted embryos seemed to keep duplicating both their DNA and their centrosomes. This observation is in stark contrast with the situation in *S. cerevisiae*, in which *cdc14-ts* mutants, when shifted to the nonpermissive temperature, were unable to exit mitosis and instead arrested in anaphase with segregated chromosomes and high Cdk1 activity (Visintin et al., 1998). To more carefully examine the influence of CeCDC-14 depletion on cell cycle progression, two types of analyses were carried out. First, embryos were dissected from worms at different times after injection of CeCDC-14–dsRNA, and the time required for these embryos to proceed through mitosis was measured. Most importantly, no cell cycle arrest could be observed at any time point (Table II). This was true even for batches of injected worms that were followed until oocytes failed to be fertilized due to depletion of the stored sperm. For embryos dissected at late time points (24–36 h after injection), our data suggested a possible increase in the length of mitosis (Table II). However, because it was difficult to measure the period between nuclear envelope breakdown and reformation accurately by DIC optics (note the relatively large standard error of the means in Table II), we also analyzed cell cycle progression in histone-GFP embryos. By filming such embryos, the time intervals between different mitotic events could be measured very accurately. As shown in Table III and Videos 1 and 2 (available at http://www.jcb.org/cgi/content/full/jcb.200202054/DC1), no significant differences in the duration of the first mitosis could be observed between wild-type and CeCDC-14–depleted embryos.

In a second series of experiments, wild-type and CeCDC-14–depleted embryos were stained with anti–phospho-histone H3 antibodies. Because the appearance and disappearance of the phospho-serine 10 epitope correlates with entry into and exit from mitosis, staining with antibodies against phospho-serine 10 has been used extensively as a marker for mitotic progression (Hendzel et al., 1997; Wei et al., 1999; Lorson et al., 2000). In both wild-type and CeCDC-14–depleted embryos, the segregated DNA masses at anaphase still exhibited substantial anti–phospho-histone H3 staining (Fig. 7, top row). However, in telophase, this staining completely disappeared, regardless of the presence or absence of CeCDC-14 (Fig. 7, second row). Both wild-type and CeCDC-14–depleted embryos then entered the next cell cycle (in the CeCDC-14–depleted situation a quadrupolar spindle was formed) and chromosomal anti–phospho-histone H3 staining reappeared again (Fig. 7, third row). In wild-type embryos at later stages, strong midbody remnant staining was observed with the anti–CeCDC-14 antibody, but the extent of anti–phospho-histone H3 staining differed between individual cells, depending on which cell cycle state they were in at the time of fixation (Fig. 7, left, bottom row). In contrast, in later CeCDC-14–depleted embryos all DNA masses were either condensed or decondensed, reflecting the fact that these embryos shared a common cytoplasm. In those embryos that displayed condensed chromosomes, most chromatin masses stained strongly positive with anti–phospho-histone H3 antibodies (Fig. 7, right, bottom row). These data show that histone H3 phosphorylation undergoes similar cyclic changes in both wild-type and CeCDC-14–depleted embryos, confirming that cell cycle progression per se is not significantly altered upon depletion of CeCDC-14 from *C. elegans* embryos. As it has recently been demonstrated that one of the kinases responsible for the mitotic phosphorylation of histone H3 on serine 10 is Aurora B (AIR-2 in *C. elegans*) (Hsu et al., 2000; Giet and Glover, 2001; Adams et al., 2001b; Crosio et al., 2002), these data also imply that the mitotic function of AIR-2 is not significantly impaired by the depletion of CeCDC-14.

Discussion

In all eukaryotic organisms, cell division needs to be spatially and temporally coordinated with chromosome segregation. Yet, different species accomplish the task of cell division in different ways. Whereas unicellular yeast divide by budding (*S. cerevisiae*) or septation (*S. pombe*), cytokinesis in animal cells is centered on the assembly of a central spindle, followed by constriction of a contractile ring (Le Goff et al., 1999; Balasubramanian et al., 2000; Glotzer, 2001). Extensive studies with budding and fission yeast have led to the elucidation of signaling pathways, termed MEN and SIN, respectively, that operate to coordinate mitotic exit and cytokinesis. In contrast, little is known about analogous regulatory circuits in animal cells. As deduced from comparative sequence analyses, animal genomes do contain apparent structural homologues of some, albeit probably not all, components of the yeast MEN/SIN pathways. Here, we have used RNAi to perform a functional analysis of potential MEN/SIN homologues in *C. elegans*. Of all genes tested, only the depletion of the CeCDC-14 phosphatase produced an embryonic lethal phenotype. Most interestingly, we could show that depletion of the CeCDC-14 phosphatase caused a severe cytokinesis failure in *C. elegans* embryos. In

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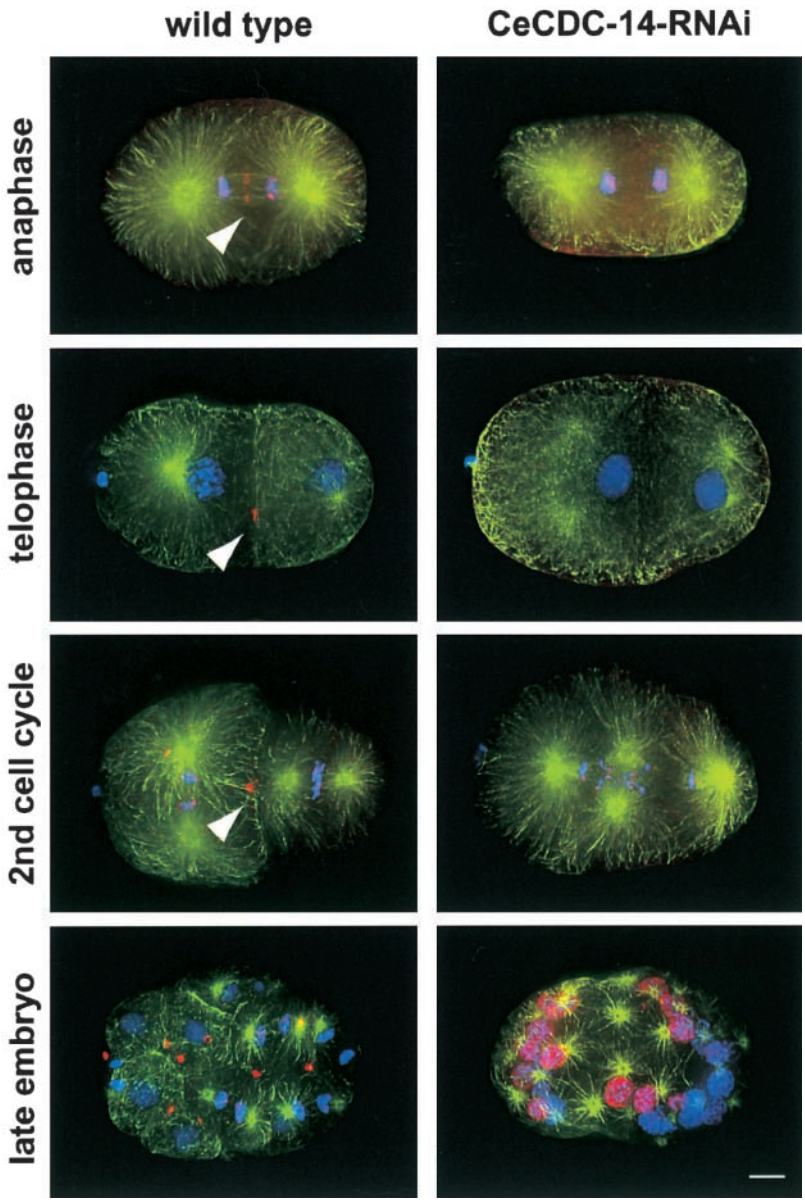

Figure 7. **The absence of CeCDC-14 does not abolish the cyclic appearance and disappearance of anti–phospho-histone H3 staining.** Wild-type and CeCDC-14–depleted embryos were stained simultaneously with anti-tubulin antibodies (green), anti–phospho-histone H3 antibodies and anti–CeCDC-14 antibodies (both in red). Rabbit antibodies against both components could be used because the chromosomal staining for phospho-histone H3 and the central spindle/midbody staining for CeCDC-14 are clearly distinct. DAPI staining is shown in blue. Arrowheads point to central spindle and midbody. Note the complete absence of midbody remnants in the CeCDC-14 embryo (right column, bottom). Bar, 5 μm.

contrast, DNA replication and segregation, as well as centrosome duplication, continued for multiple cell cycles.

Depletion of CeCDC-14 but not other MEN/SIN homologues causes embryonic lethality

Surprisingly, our RNAi-based survey of potential *C. elegans* MEN/SIN homologues revealed an embryonic requirement only for the CeCDC-14 phosphatase. Several factors may contribute to explain this finding. First, two of the MEN/SIN components analyzed (Dbf2p and Mob1p) have more than one potential homologue in the *C. elegans* genome, raising the possibility of redundancy. In these cases, it might be necessary to simultaneously deplete all potential homologues in order to observe a phenotype. Second, it is possible that some of the MEN/SIN components serve to fulfill requirements specific to yeast cell budding or fission, respectively, and these functions may not have been conserved during evolution of metazoan organisms. Third, whether or not a particular gene product is required for cell cycle progression may depend on the developmental context. Consistent with this possibility, we have observed that depletion of two *C. elegans* kinases structurally related to MEN/SIN components (Dbf2p of *S. cerevisiae* and Sid1p of *S. pombe*) produced an F1 arrest at the L1 stage and degeneration of the germ line, respectively.

CeCDC-14 regulates cytokinesis in *C. elegans* embryos

Our studies provide strong evidence for a key role of the metazoan Cdc14 phosphatase in cytokinesis. As a result of this critical requirement, *C. elegans* embryos depleted of CeCDC-14 display early embryonic lethality. The phenotype of embryos derived from CeCDC-14–dsRNA-injected worms indicates that CeCDC-14 is important for the regulation of cytokinesis per se, independent of a possible role in cell cycle control. Previous studies on the budding yeast Cdc14p protein had not revealed a direct role for this phosphatase in cytokinesis. However, because *cdc14-ts* mutants arrest at late anaphase, any requirement for Cdc14p at later

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stages of the cell cycle might have escaped detection. In the case of fission yeast, some cytokinesis defects could be observed in the absence of Clp1p/Flp1p, particularly in conjunction with mutations of other SIN components (Cueille et al., 2001). Thus, this phosphatase may play a role in the regulation of cytokinesis not only in animal cells but also in yeast.

CeCDC-14 depletion does not affect the embryonic *C. elegans* cell cycle

In both *S. cerevisiae* and *S. pombe*, Cdc14p and Clp1p/Flp1p have been implicated in the regulation of Cdk1 activity, albeit at different stages of the cell cycle. In striking contrast, in *C. elegans* embryos depleted of CeCDC-14, the nuclear cycles continued with similar timing as in wild-type embryos. Taken at face value, this result indicates that the *C. elegans* Cdc14 phosphatase is required for cytokinesis but not for other aspects of cell cycle control. Of course, it is difficult to rigorously exclude that some residual CeCDC-14 protein, at levels undetectable by immunofluorescence microscopy, might be sufficient to fulfill a potential cell cycle function. In an attempt to maximize the depletion of CeCDC-14, we have also analyzed embryos that were dissected after prolonged incubation of injected worms. This has been reported to enhance depletion of some proteins (Rappleye et al., 2002), but in our experiments did not give rise to additional phenotypes. The vast majority of treated embryos was defective in both polar body extrusion and cytokinesis, and yet progressed through the cell cycle with near-normal kinetics. At the very least, this suggests that embryonic cell cycle progression is much less dependent on Cdc14 activity than cytokinesis.

It is important to bear in mind that embryonic cell cycles may lack certain controls that are operating at later developmental stages. For instances, embryonic cycles appear to be devoid of checkpoint mechanisms, as indicated by their inability to arrest in response to nocodazole (Hyman and White, 1987; Kitagawa and Rose, 1999). Thus, although the direct regulation of cytokinesis may well be the only essential function of CeCDC-14 in the embryonic cycles, additional functions may be required at larval stages. We cannot presently test this possibility, because the CeCDC-14–depleted embryos die before hatching. Thus, to further elucidate a potential role for CeCDC-14 in the regulation of somatic cell cycles, temperature-sensitive mutants will be required.

Depletion of CeCDC-14 interferes with central spindle assembly

In embryos depleted of CeCDC-14, bundled midzone microtubules did not assemble, and the kinesin-related motor ZEN-4 failed to localize to the spindle midzone. This behavior of ZEN-4 is different from that observed in embryos depleted for the INCENP protein ICP-1, in which ZEN-4 could locate to the central spindle, albeit transiently (Kaitna et al., 2000). The phenotype observed here could be explained in two ways: first, it is possible that CeCDC-14 regulates an upstream factor essential for central spindle formation. In this scenario, the failure of ZEN-4 to localize to the central spindle in CeCDC-14–depleted embryos would simply reflect the absence of stable midzone microtubules. Alternatively, ZEN-4, and/or its partner CYK-4, could themselves be regulated by CeCDC-14. Specifically, one could argue that ZEN-4 and/or CYK-4 need to be dephosphorylated in order to localize to the forming central spindle, and that in their absence, microtubule bundles might disassemble. To test this second possibility, we have phosphorylated recombinant ZEN-4/CYK-4 complexes by Cdk1-cyclin B in vitro, and then used these phosphoproteins as substrates for CeCDC-14. However, although the recombinant phosphatase readily dephosphorylated histone H1, no dephosphorylation of either ZEN-4 or CYK-4 could be observed (M. Mishima, U. Gruneberg, and M. Glotzer, personal communication). In future studies, it will thus be interesting to consider additional candidate substrates of CeCDC-14. These might include microtubule-stabilizing factors or other regulatory proteins involved in the process of central spindle formation.

Materials and methods

RNA-mediated interference

For CeCDC-14–RNAi, full-length inserts of ESTs yk506f9, yk573g10, and yk643d10 were amplified using primers CMO24 (TTGTAAAACGACGGCCAG) and CMO25 (CATGATTACGCCAAGCTC) which anneal to the vector backbone of pBluescript SK−. The resulting PCR products were used as templates for in vitro transcription using Ribomax T7- and T3-in vitro transcription kits (Promega). Double-stranded RNA was produced as described (Fire et al., 1998), diluted with injection buffer (6.6 mM K-phosphate, pH 7.5, 1 mM K-citrate, pH 7.5, 0.66% PEG6000), and injected into the gonads of young adult N2 hermaphrodites at a final concentration of 3–5 μg/μl. Injected worms were kept at 20°C and analyzed at the indicated time points after injection. The embryos of injected worms showed embryonic lethality, but no defects were observed in the germ line of the injected worms. For immunofluorescence microscopy worms were harvested between 20 and 28 h after injection, unless indicated otherwise. For ZEN-4–RNAi and AIR-2–RNAi the inserts of EST yk35d10 and yk665d12, respectively, were amplified as above, TA-cloned into feeding vector L440, and transformed into HT115 bacteria. These transformed bacteria were then used for RNAi feeding of N2 L4 larvae (Timmons et al., 2001). Worms were dissected after a 20-h incubation on feeding plates at 15°C.

For the depletion of potential *C. elegans* MEN/SIN homologues other than CeCDC-14, the following ESTs were used for preparing dsRNA or feeding constructs, as described above: yk157e4, yk249f3 (R11G1.4), yk467f11 (T12B3.4), yk8f12 (T19A5.2), yk50a8 (T20F10.1), yk462g7, and yk373c2 (F09A5.4). F38H4.10 and C33F10.2 were amplified from *C. elegans* cDNA and TA cloned either into L440 for feeding experiments or into pCRII-TOPO (Invitrogen). For in vitro transcription, the inserts were amplified with M13-FW and RV-primers and transcribed using T7 and SP6 polymerase. All ESTs used in this study were provided by Dr. Yuji Kohara (National Institute of Genetics, Mishima, Japan).

DIC filming of worm embryos

All filming of embryos was conducted at 20°C. Worms were dissected in a drop of PBS on an 18 × 18-mm coverslip and mounted onto a 2% agar pad. The coverslip was sealed with silicon glue and embryos were recorded with a 63×/1.4 objective using DIC optics on a Zeiss Axiovert S 100 microscope equipped with a JAI CV-M50 camera and Image-Pro Express 4.0 software. Single frames were then exported into Adobe Photoshop.

Time-lapse recording of ZEN-4–GFP and histone H1-GFP embryos

For real-time recordings of ZEN-4–GFP in *C. elegans* embryos, strain MG170 (*zen-4* [or *153ts*]); (*xsEx6* [*zen-4–GFP*]) (Kaitna et al., 2000) was used. This strain was propagated at 25°C. Untreated or RNAi-injected MG170 worms were dissected as described above, and time-lapse recordings of GFP fluorescence were carried out as described previously (Jantsch-Plunger et al., 2000), using a Zeiss Axioplan 2 microscope with a piezo stepper (Physik Instrumente), a CoolSnap camera for image acquisition and Metamorph software. The exposure time for GFP fluorescence was 100 ms, and six frames, 2 μm apart, were taken every 15 s. Time-lapse recordings of histone H2B-GFP embryos were performed in a similar manner, with the exception that only two frames, 1.5 μm apart, were taken every 6 s in order to increase temporal resolution. Filming of his-

tone-GFP embryos was carried out at 25°C. For data analysis, each stack of GFP images was projected onto a single plane, and the projections were processed for figures using Adobe Photoshop.

Phosphatase assays

Full-length CeCDC-14 was cloned into pQE32 (QIAGEN) and purified according to standard procedures using Ni-NTA agarose (QIAGEN). The protein was eluted with 20 mM Tris-Cl, pH 8.0, 300 mM NaCl, 200 mM imidazole, and the imidazole subsequently removed by passing the eluate over a 5-ml HiTrap desalting column (Amersham Biosciences). Purified CeCDC-14 was stored at –80° in 25 mM Hepes, pH 7.3, 300 mM NaCl, 1 mM EDTA, 1 mM DTT, 10% glycerol. Phosphatase activity was measured in reaction buffer consisting of 50 mM imidazole, pH 6.9, 1 mM DTT, 1 mM EDTA as described (Taylor et al., 1997), using 1 mg/ml para-nitrophenyl phosphate (Sigma-Aldrich) and 200–6,400 ng of enzyme. Control reactions were carried out using the phosphatase-dead CeCDC-14 (cysteine 295 mutated to serine).

Antibodies

For the production of CeCDC-14-specific antibodies, rabbits were immunized with a GST–CeCDC-14 aa 352–605 fusion protein expressed in *E. coli*. Antibodies were affinity-purified using a 6His-CeCDC-14 fusion protein coupled to Affi-Gel 10 and used at a final concentration of 0.25 μg/ml.

The antibodies specific for ZEN-4 and AIR-2 have been described (Jantsch-Plunger et al., 2000; Kaitna et al., 2002). Tubulin was stained with the mouse monoclonal antibody DM1A (Sigm-Aldrich). Antibodies recognizing phospho-histone H3 (serine10) were obtained from Upstate Biotechnology. Affinity-purified sheep–anti-GFP antibody was a gift from Dr. Francis Barr (Max-Planck Institute for Biochemistry). Anti–PGL-1 antibodies and antibodies against PAR-2 and PAR-3 were provided by Drs. Susan Strome (Indiana University, Bloomington, IN) and Ken Kemphues (Cornell University, Ithaca, NY), respectively.

Insect cell expression

For baculovirus expression, full-length C17G10.4c was amplified by RT-PCR from total worm cDNA using an Advantage RT-for-PCR kit (CLONTECH Laboratories, Inc.), TA-cloned into pCRII-TOPO (Invitrogen) and subcloned into the baculovirus transfer vector pAcSG2 (Becton-Dickinson). Recombinant baculoviruses were generated by cotransfecting Sf9 cells with the C17G10.4c transfer vector and BaculoGold-DNA (Becton-Dickinson) according to the manufacturer's protocols.

Immunoblotting

Total worm lysate was prepared by resuspending a worm pellet of 100 μl in 200 μl urea-buffer (Knop et al., 1996). The lysate was heated at 65°C for 10 min and 5 μl were applied per lane of an SDS-PAGE minigel. The separated proteins were transferred to Protran membrane (Schleicher and Schuell) and probed with affinity-purified anti–CeCDC-14 antibodies at a concentration of 0.25 μg/ml. For analysis of untagged recombinant CeCDC-14 protein, baculovirus-infected Sf9 cells were harvested 48 h postinfection with the C17G10.4c-virus. The cell pellet was lysed at 4×10^6 cells/ml in 50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 0.5% IGEPAL, 1 mM Pefabloc (Roche), and a protease inhibitor cocktail (Roche). The lysate was centrifuged for 10 min at 14,000 rpm in a table top centrifuge and the supernatant diluted 1:10 with PBS. 5 μl was loaded per lane of an SDS-PAGE minigel and processed as described above.

Immunofluorescence microscopy

For staining of phospho-histone H3, ZEN-4, AIR-2, and CeCDC-14, embryos were freeze cracked and fixed in –20°C methanol for 20 min as described (Jantsch-Plunger et al., 2000). Phalloidin staining was performed using a modified version of the published protocol (Strome, 1986). The fixation/staining solution was 4% paraformaldehyde, 50 mM Pipes, pH 6.8, 5 mM EGTA, 300 nM FITC-phalloidin. For P granule staining embryos were fixed for 10 min in cold methanol followed by 10 min in cold acetone ass described (Kawasaki et al., 1998). Stacks of immunofluorescence images 0.2 μm apart were collected using a Deltavision microscope (Applied Precision) and a 100×/1.4 oil objective. The three-dimensional stacks (typically 35–45 planes) were computationally deconvolved, projected onto a single plane and further processed for figures in Adobe Photoshop.

Online supplemental material

Fig. S1 (available at http://www.jcb.org/cgi/content/full/jcb.200202054/DC1) documents the phenotypes observed upon depletion of the putative Dbf2p homologue T20F10.1 and the putative Sid1p homologue T19A5.2.

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