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The *Caenorhabditis elegans* polarity gene *ooc-5* encodes a Torsin-related protein of the AAA ATPase superfamily

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SUMMARY

The PAR proteins are required for polarity and asymmetric localization of cell fate determinants in *C. elegans* embryos. In addition, several of the PAR proteins are conserved and localized asymmetrically in polarized cells in *Drosophila*, *Xenopus* and mammals. We have previously shown that *ooc-5* and *ooc-3* mutations result in defects in spindle orientation and polarity in early *C. elegans* embryos. In particular, mutations in these genes affect the re-establishment of PAR protein asymmetry in the P_1 cell of two-cell embryos. We now report that *ooc-5* encodes a putative ATPase of the Clp/Hsp100 and AAA superfamilies of proteins, with highest sequence similarity to Torsin proteins; the gene for human Torsin A is mutated in individuals with early-onset torsion dystonia, a neuromuscular disease. Although Clp/Hsp100 and AAA family proteins have roles in diverse cellular activities, many are involved in the assembly or disassembly of proteins or protein complexes; thus, OOC-5 may function as a chaperone. OOC-5 protein co-localizes with a marker of the endoplasmic reticulum in all blastomeres of the early *C. elegans* embryo, in a pattern indistinguishable from that of OOC-3 protein. Furthermore, OOC-5 localization depends on the normal function of the *ooc-3* gene. These results suggest that OOC-3 and OOC-5 function in the secretion of proteins required for the localization of PAR proteins in the P_1 cell, and may have implications for the study of torsion dystonia.

Key words: *Caenorhabditis elegans*, Polarity, Asymmetric division, Endoplasmic reticulum

INTRODUCTION

Asymmetric divisions generate cellular diversity during development in a wide range of organisms. During intrinsically asymmetric divisions, a polarized parent cell divides to produce daughters that are different. For such a division to occur, the division plane, which is specified by the position of the mitotic spindle, must be aligned with the axis of polarity (Bowerman and Shelton, 1999; Doe and Bowerman, 2001; Hawkins and Garriga, 1998; Jan and Jan, 2000; Rose and Kemphues, 1998).

Recent work has implicated a conserved set of proteins as functioning in cellular polarity and/or asymmetric divisions in several organisms. The PAR proteins were originally identified in *C. elegans* as being essential for establishment of polarity and partitioning of cell fate determinants (Kemphues et al., 1988). PAR-3 and PAR-6, both PDZ domain proteins, and an atypical protein kinase C, PKC-3, are all localized to the anterior cortex of asymmetrically dividing cells in early embryos (Etemad-Moghadam et al., 1995; Hung and Kemphues, 1999; Tabuse et al., 1998). Homologs of these proteins localize to the animal pole of mature oocytes and embryos in *Xenopus* (Nakaya et al., 2000), to the tight junctions of epithelial cells in *Xenopus* and mammals (Izumi et al., 1998; Nakaya et al., 2000; Suzuki et al., 2001), and to the apical surface of epithelial cells and asymmetrically dividing neuroblasts in *Drosophila* (Kuchinke et al., 1998; Petronczki and Knoblich, 2001). Several studies have shown that PAR-3, PAR-6 and PKC-3 can bind to each other, and that they are interdependent upon each other for asymmetric localization (Hung and Kemphues, 1999; Izumi et al., 1998; Joberty et al., 2000; Lin et al., 2000; Petronczki and Knoblich, 2001; Qiu et al., 2000; Suzuki et al., 2001; Tabuse et al., 1998). Disruption of PAR-3 or PAR-6 in cultured mammalian epithelial cells results in a loss of junctional complexes and disruptions in surface polarity (Joberty et al., 2000; Suzuki et al., 2001). Similarly, loss of function of the *Drosophila* PAR-3 and PAR-6 homologs disrupts epithelial integrity and causes misorientation of asymmetric divisions in neuroblasts (Kuchinke et al., 1998; Petronczki and Knoblich, 2001). Finally, the PAR-6 protein was recently found to associate with CDC42 in mammalian cells; inhibition of CDC42 function disrupts polarity in mammals and in *C. elegans* embryos (Gotta et al., 2001; Joberty et al., 2000; Kay and Hunter, 2001; Lin et al., 2000; Qiu et al., 2000). Thus, it is clear that an asymmetric distribution of the PAR-3/PAR-6/PKC-3 complex is characteristic of many types of polarized cells, and some aspects of its function in polarity may be conserved as well. However, the precise means by which these proteins are localized to their appropriate cortical domains remains to be elucidated.

C. elegans is an excellent animal in which to study the

mechanisms that establish polarized protein domains. The early embryo is characterized by a series of asymmetric divisions which produce blastomeres with different cellular fates and segregate the germline potential to a single precursor. The PAR proteins (mutations in which are partitioning defective) are required for generating asymmetry during these divisions (Bowerman and Shelton, 1999; Kemphues and Strome, 1997; Rose and Kemphues, 1998). PAR proteins become asymmetrically localized at the cortex in response to a cue from the sperm aster, which defines the posterior pole of the embryo (Goldstein and Hird, 1996; Wallenfang and Seydoux, 2000). PAR-3, PAR-6 and PKC-3 localize to the anterior cortex, while PAR-2 and PAR-1 localize to the posterior. PAR-2 and the PAR-3 are interdependent for their localization; that is, mutation in one results in uniform cortical distribution of the other in one-cell embryos. The localization of PAR-1 depends on proper formation of the PAR-2 and PAR-3 domains (Boyd et al., 1996; Etemad-Moghadam et al., 1995; Guo and Kemphues, 1995; Hung and Kemphues, 1999; Tabuse et al., 1998). PAR function is required during the one-cell stage for the differential segregation of a number of molecules such as PIE-1 and P granules to the daughter cells, and for setting up differences in cell cycle rates and division orientation of the daughter cells (Bowerman and Shelton, 1999; Kemphues and Strome, 1997; Rose and Kemphues, 1998). At the two-cell stage, PAR-2 and PAR-1 are initially present around the entire cortex of P_1. Before mitosis, asymmetry is re-established, with P granules, PAR-2 and PAR-1 becoming restricted to the posterior pole of P_1, while PAR-3 localizes to the anterior cortex. Rotation of the nuclear-centrosome complex aligns the spindle with this axis of polarity, ensuring a second asymmetric division. The cue for refining PAR domains at the two-cell stage remains to be identified.

We have previously shown that *ooc-5* is required for polarity in the two- cell *C. elegans* embryo (Basham and Rose, 1999). Worms homozygous for mutations in *ooc-5* have an abnormal germline morphology and produce oocytes and embryos that are reduced in size. The mutations are also maternal effect lethal, and the embryos produced (referred to hereafter as *ooc-5* embryos) have unique defects in the localization of PAR proteins. In *ooc-5* mutant embryos, PAR proteins are localized normally at the one-cell stage, but are mislocalized in most two-cell embryos. Specifically, PAR-3 extends too far towards the posterior in P_1, and is often found around the entire cortex; PAR-2 and PAR-1 localization to the posterior is concomitantly reduced or absent, and P granules are also mislocalized at the two-cell stage. In addition, *ooc-5* embryos show partial defects in nuclear rotation at the one-cell stage, and failure in nuclear rotation in the P_1 cell. Double mutant analysis showed that mislocalization of PAR-3 contributes to the failure of P_1 nuclear-rotation, but also revealed a PAR-independent effect on spindle position in one and two-cell embryos. Thus, *ooc-5* plays roles both in spindle orientation and in PAR protein localization. We and others showed that mutations in *ooc-3* produce a nearly identical phenotype to that of *ooc-5*, and thus these genes probably function in a common mechanism to maintain or re-establish polarity in P_1 (Basham and Rose, 1999; Pichler et al., 2000).

In this work, we report that *ooc-5* encodes a putative ATPase of the AAA family of proteins, which has significant sequence similarity to the Torsin proteins. OOC-5 co-localizes with a marker for the endoplasmic reticulum in all cells of the early embryo, and *ooc-3* is required for this localization. These results suggest a role for OOC-5 in the secretion of plasma membrane proteins that function in setting up PAR protein domains.

MATERIALS AND METHODS

Culture conditions and strains

C. elegans strains were cultured using standard techniques (Brenner, 1974). All strains were derived from the wild-type Bristol strain N2. N2 worms were used as wild-type controls. Strains containing the following previously described mutations or rearrangements were used: LGII, *ooc-5(it144)*, *ooc-5(it145)*, *ooc-5(it146) unc-4 (e120)*, *ooc-3 (mn241)*, *mnC1*, *mnDf67*, *mnDf85* and *mnDf61*. Strains were obtained from the Caenorhabditis Genetics Center, the Kemphues lab or were constructed in the Rose laboratory. For all experiments, worms were grown at 20°C.

Positional cloning and germline transformation

The deletions *mnDf61* and *mnDf85* were tested for complementation of *ooc-5(it145*) using standard genetic crosses. To map the right-hand break point of *mnDf85*, a PCR-based approach was used (McKim et al., 1994). Homozygous *mnDf85* deletion embryos were picked as unhatched embryos at least 24 hours after being laid by deletion-heterozygote mothers and prepared for PCR as in Williams et al. (Williams et al., 1992). PCR reactions were performed to test for the presence of cosmid sequences, using at least five sets of deletion embryos per primer set. Reactions were carried out using primers near the right ends of cosmids F07H5, T14D7 and T21B10. PCR amplification gave products of the predicted size using T14D7 and T21B10 primers, but failed to give a product using F07H5 primers, indicating that *mnDf85* breaks between the right ends of F07H5 and T14D7. The left breakpoint of mnDf61 is based on Kramer et al. (Kramer et al., 1990).

Cosmids (kindly provided by the *C. elegans* Genome Consortium) were co-injected with the pRF-4 plasmid carrying the dominant marker *rol-6(su1006)* at a concentration of 30:70 μg/ml into *ooc-5(it145) unc-4(e120)/mnC1* hermaphrodites using standard procedures (Mello and Fire, 1995). To assay for rescue, individual Roller Unc progeny from heritable lines were examined for their ability to produce wild-type size embryos that hatched. Rescue was defined as the ability of an *ooc-5* mutant hermaphrodite carrying a cosmid to produce some wild-type sized embryos that hatched. Cosmid fragments were tested for rescue either as gel purified pieces (>10 kb) or subcloned fragments.

Molecular characterization of the *ooc-5* gene

Plasmids containing cDNA yk504f1, yk643a1 and yk452e10 (kindly provided by Y. Kohara) were isolated using standard procedures (ExAssist, Stratagene). To test if the predicted F44G4.1 exons 1-4 and exons 5-10 are part of the same transcript, PCR reactions were carried out using a mixed stage cDNA library (Kraemer et al., 1999) and primers designed to amplify either exons 3-4, exons 4-5 or exons 5-8. Products were reproducibly amplified that corresponded to either gene, but no products that included sequences from both were amplified. To characterize the 5′ end of *ooc-5*, PCR products were amplified using an SL1, SL2, or library-specific primer in combination with an *ooc-5* specific 3′ primer. To determine the 3′ end of *ooc-5*, PCR amplification was performed using a 5′ primer corresponding to exon 4 and a 3′ primer specific to the cDNA library. PCR products were cloned into the pGEMT Easy vector (Promega). The SL1 clone, 3′ clone and the yk504f1 cDNA were sequenced in their entirety.

To sequence *ooc-5* mutant alleles, genomic DNA was isolated from wild-type and *ooc-5* mutant hermaphrodites, and amplified using primers flanking the *ooc-5*-coding region (Boehringer Mannheim's

Expand and High Fidelity Kits). PCR products were cloned into pGEMT Easy and four independent PCR reaction products were sequenced for each *ooc-5* mutant allele. All sequencing was carried out by Davis Sequencing (Davis, CA). Sequence analysis was performed using DNA Star software.

For RNA interference experiments, cDNAs yk643a1 and yk452e10 (F44G4.1), or a 1.2 Kb *Eco*R1 fragment subclone of yk504f1 (containing sequence identity to C18E9.11 only) were linearized and used for in vitro transcription of sense and antisense RNA (Ambion MEGAscript). Equal amounts of sense and antisense RNA were mixed with 3× injection buffer, heated to 90°C for 10 minutes, then cooled to room temperature (Fire et al., 1998). The annealed dsRNA (1 mg/ml) was injected into the gonad of wild-type hermaphrodites; F_1 progeny were scored for hatching, and those that hatched scored for egg size and lethality of the F_2 embryos.

Antibodies and immunolocalization

A fragment of the *ooc-5* gene encoding the C-terminal 120 amino acids was cloned into the *Eco*R1 site of the pGEX4T-3 vector (Pharmacia). The GST:OOC-5 fusion protein was expressed in bacteria and column purified on Glutathione Sepharose beads (Pharmacia) using standard procedures and injected into two rabbits (#75064 and #75068) by the U.C. Davis Animal Resources Service. Polyclonal antisera was affinity purified against GST: OOC-5 protein immobilized on nitrocellulose strips. Antibodies were eluted with 0.2 M glycine HCl, 1 mm EGTA pH 2.8 and immediately neutralized to pH 7.4 with 1 M Tris base, and glycerol and BSA were added to a final concentration of 10% and 1 mg/ml respectively. Purified antibodies from both rabbits gave the same staining pattern; #75068 antibodies gave a stronger signal and were used for all data shown.

Whole-worm extracts from approximately 250 gravid adult worms were resolved on 10% SDS-polyacrylamide gels, and subjected to western blotting using standard procedures (Harlow and Lane, 1988). Blots were incubated with anti-OOC-5 antibodies (1:1,000 in TBST) and visualized using the ECL detection system (Amersham). Blots were stripped in 100 mM β-mercaptoethanol; 2% SDS; 62.5 mM Tris-HCl pH 7.5 for 15 minutes at 65°C and then re-probed with the anti-α-tubulin antibody DM1A (1:5000; Sigma).

For immunolocalization, embryos were released from hermaphrodites on poly-lysine coated slides in M9 buffer and permeabilized by freeze-fracture (Miller and Shakes, 1995). For all antigens except GLP-1, embryos were then fixed in 75% methanol, 3.7% formaldehyde and 0.5× PBS (–20°C, 15 minutes) followed by absolute methanol (–20° C, 15 minutes). For GLP-1 staining, embryos were fixed for 15 minutes in –20°C absolute methanol. Slides were then rehydrated in PBST (room temperature, 15 minutes), blocked with 30% normal goat serum/PBST (15 minutes) and incubated with primary antibodies diluted in PBST (rabbit anti-OOC-5 1:10; rabbit anti-OOC-3 1:250; mouse anti-HDEL 1:20; mouse anti-DM1A 1:50; rabbit anti-GLP-1 1:10; 4°C, overnight). Slides were washed in PBST (four times for 5 minutes), incubated with secondary antibodies diluted 1:100 in PBST (fluorescein conjugated anti-rabbit and rhodamine conjugated anti-mouse (Chemicon International); room temperature, 1 hour) and washed in PBST (four times for 5 minutes, one wash contained DAPI (4′,6-diamidino-2-phenylindole dihydrochloride)). Specimens were mounted with Vectashield (Vector Laboratories) and viewed using either epifluorescence or confocal microscopy. OOC-3, HDEL and GLP-1 antibodies were kindly provided by Tony Hyman (Max Planck Institute, Dresden, Germany) Sean Munro (MRC, Cambridge, UK) and Judith Kimble (HHMI, University of Wisconsin) respectively. Other antibodies against ER antigens were tested for cross reactivity with *C. elegans* using the same fixation as above or absolute methanol only; antibodies tested were anti-KDEL, anti-grp78 (StressGen Biotechnologies Corp.), anti-mp30 and anti-SR-beta (kindly provided by Peter Walter, HHMI, University of California, San Francisco).

RESULTS

Molecular isolation of *ooc-5*

The *ooc-5* gene functions in the formation of normal oocytes and the establishment of polarity in the two-cell embryo. As the first step towards elucidating the role of *ooc-5* at the molecular level, we identified the gene using a positional cloning approach (Fig. 1). Meiotic recombination and complementation tests with deletions narrowed the potential *ooc-5*-encoding region to seven overlapping cosmids (Fig. 1A). Cosmid C18E9 rescued *ooc-5* mutants in germline transformation experiments, while cosmids flanking C18E9 did not. A 6.3 kb fragment of C18E9 also rescued *ooc-5* mutants (Fig. 1B); this fragment was initially predicted to contain a single open reading frame (ORF) called F44G4.1 (The *C. elegans* Sequence Consortium, 1998) (note that cosmid F44G4 begins in exon 4 of this ORF). However, analysis of cDNAs indicate that these predicted exons actually correspond to two separate genes (Fig. 1C) and these have been renamed C18E9.11 and F44G4.1 (WormBase) (Stein et al., 2001). The short distance separating C18E9.11 and F44G4.1 is consistent with these two genes being part of an operon (Blumenthal and Steward, 1997).

RNA interference was used to determine which ORF corresponds to *ooc-5* (Fire et al., 1998). Wild-type worms injected with dsRNA from C18E9.11 exons produced progeny that laid small *ooc-5* embryos that failed to hatch, while worms injected with dsRNA from F44G4.1 exons produced progeny that were viable and laid normal embryos. These results indicate that *ooc-5* corresponds to C18E9.11. This was confirmed by rescuing *ooc-5* mutants with a 4.4 kb fragment of C18E9 containing only this ORF (Fig. 1B). Finally, sequence analysis of the three *ooc-5* mutant alleles revealed molecular lesions within the C18E9.11 exons (Fig. 1D). Taken together, these results demonstrate that the *ooc-5* gene corresponds to C18E9.11.

Amplification of cDNA from mixed stage libraries using the *C. elegans* SL1 primer and an *ooc-5* specific 3′ primer showed that the SL1 sequence is trans-spliced four nucleotides upstream of the first ATG in *ooc-5*. In addition, the first 18 nucleotides of predicted intron 1 were present in the SL1-*ooc-5* PCR product but were absent in PCR products amplified using a 5′ primer specific to the library, and in the yk504f1 cDNA. These results indicate that *ooc-5* can be alternatively spliced.

OOC-5 encodes a putative AAA+ ATPase related to the Torsin family of proteins

Conceptual translation of the SL1 spliced version of *ooc-5* results in a 357 amino acid protein with a predicted molecular mass of ~39 kDa; the alternatively spliced version would be six amino acids smaller (Fig. 1D). The N-terminal region of both isoforms of OOC-5 contains a stretch of hydrophobic amino acids, the first 18 of which are predicted to form a cleavable signal peptide (predicted using PSORT II) (Nakai and Kanehisa, 1992). Searches for protein motifs using the SMART (Simple Modular Architecture Research Tool) (Schultz et al., 2000) and Pfam (Bateman et al., 1999) databases indicate that OOC-5 is a member of the AAA family of ATPases (ATPases associated with a variety of cellular activities; Fig. 1D). AAA ATPases have been shown to play

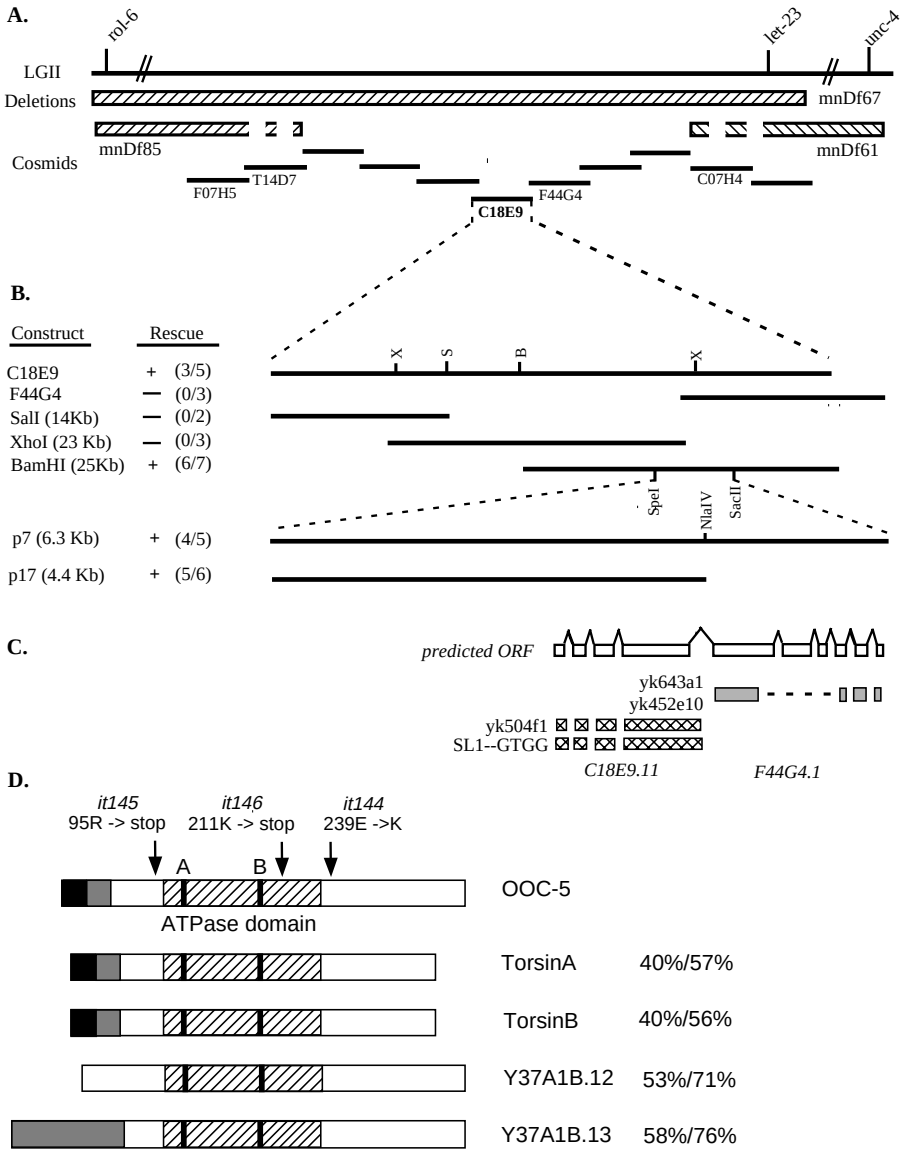

Fig. 1. Molecular cloning of the *ooc-5* locus. (A) Schematic diagram of the genetic and physical maps in the *ooc-5* region. The top line shows the *rol-6 unc-4* interval of the genetic map of chromosome II (LGII). Below it are shown deficiencies, with the region deleted indicated by hatched bars and the uncertainty in breakpoint shown by broken bars. The physical map of overlapping cosmids (black lines) is shown from left to right: F07H5, T14D7, T07D4, B0457, T21B10, C18E9, F44G4, F37B12, T24B8, C07H4, T08E2. (B) Transformation rescue of *ooc-5* mutants. The name of the cosmid or subfragment tested (construct) for rescue is shown on the left, with a plus indicating rescue and the number of lines rescued/total tested shown in parentheses. The extent of each construct is shown on the right. (C) Predicted open reading frames (ORFs) and cDNAs corresponding to the two smallest rescuing fragments. The predicted exons (white boxes) and introns for F44G4.1 (predicted by the *C. elegans* Genome Project) are shown, with the corresponding regions of identity with cDNAs indicated below. The available sequence tags of yk643a1 and yk452e10 (gray boxes; Wormbase) were used to align these cDNAs, while the SL1 5′ clone, a 3′ clone and yk504f1 (cross-hatched boxes) were sequenced in their entirety. The 3′ cDNA clone includes 168 nucleotides of predicted intron 4. The yk504f1 sequence has 172 nucleotides of predicted intron 4 and a stretch of A residues; this is followed by sequences with identity to T23E1.2, an unrelated ORF, which suggests it is a hybrid cDNA. (D) Schematic representation of OOC-5 domains and homology with other Torsin family members. Black boxes represent predicted signal peptides and gray boxes are additional hyrdrophobic regions. The hatched regions indicate the AAA+ ATPase domain, with the position of the Walker A (A) and Walker B (B) motifs labeled. The larger isoform of OOC-5 is shown; the smaller isoform is missing amino acids 27-32.The amino acid changes associated with the three *ooc-5* mutations are indicated by arrows. Percent identity/similarity between OOC-5 and other torsin family members is indicated to the right of each protein; the regions of similarity begin after the hydrophobic domain (amino acid 49 of OOC-5) and continue for the length of the entire proteins. The GenBank Accession Numbers for these sequences are: OOC-5, Z70034 (Accession Number is for cosmid C18E9; the large OOC-5 isoform is C18E9.11b, the short isoform is C18E9.11a); Torsin A, NM_000113; Torsin B, AF317129; Y37A1B.12, CAA19495; and Y37A1B.13, CAA19484.

roles in many different cellular processes, including protein folding, proteolysis and membrane trafficking (Neuwald et al., 1999; Vale, 2000). BLAST searches of the NCBI database (Altschul et al., 1997) reveal that OOC-5 shares highest similarity with two predicted *C. elegans* proteins, Y37A1b.12 and Y37A1b.13, and the Torsin A and Torsin B proteins of mouse and human (Fig. 1D). The gene for Torsin A (*DYT1*) is mutated in individuals with early-onset dystonia, a neuromuscular disease (Ozelius et al., 1997; Ozelius et al., 1998). OOC-5 and the human Torsins have a similar domain organization and the sequence identity (40%) extends over most of the length of the proteins (Fig. 1D). Therefore, OOC-5 is a Torsin-related protein. Although the function of Torsin proteins is not known, within the AAA family, OOC-5 and the Torsins are most similar to the Clp/Hsp100 ATPases (Apweiler et al., 2001; Ozelius et al., 1997). Like many AAA proteins, the Clp/Hsp100 family members function as chaperones in the unfolding or folding of proteins (Schirmer et al., 1996).

OOC-5 co-localizes with an ER marker in early blastomeres

To determine the subcellular localization of OOC-5 in early embryos, polyclonal antisera against the C-terminal region of the OOC-5 protein were generated. On western blots, affinity-purified OOC-5 antibodies recognize a band of approximately 41 kDa in extracts of wild-type egg-bearing hermaphrodites, which is absent from protein extracts of *ooc-5(it145)* mutant worms (Fig. 2). These antibodies were used to perform OOC-

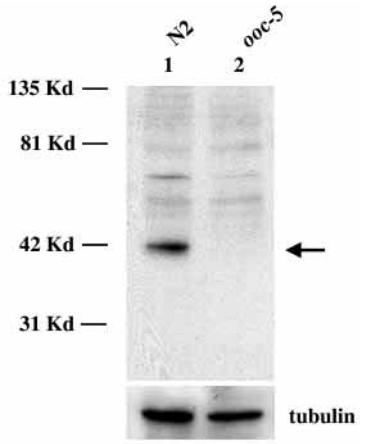

Fig. 2. Western blot analysis of OOC-5 protein. Blot of whole worm extracts from gravid adult wild-type worms (lane 1) or *ooc-5 (it145)* homozygous mutant worms (lane 2) probed with anti-OOC-5 antibodies. The arrow indicates the expected position of the OOC-5 protein in the mutant lane. Faint higher molecular weight bands seen in both the wild-type and *ooc-5* lanes were also seen in pre-immune controls (not shown). The same blot was probed with anti-α tubulin antibodies as a loading control.

5 localization studies in wild-type embryos. The localization patterns described below were completely absent in *ooc-5 (it145)* mutant embryos (Fig. 3), showing that the patterns are specific for OOC-5.

OOC-5 localization was observed in all cells of early wild-type embryos, in a pattern that changed with the cell cycle. In early one-cell interphase and prophase embryos, OOC-5 was enriched around the rim of both pronuclei, as well as being dispersed throughout the cytoplasm in a slightly reticular pattern (Fig. 3A-C). A similar distribution was seen in two-cell interphase and prophase embryos. Double labeling with antibodies against tubulin showed that regions of more concentrated OOC-5 staining corresponded to the areas of the microtubule asters (Fig. 3G-I). During metaphase in one- and two-cell embryos, OOC-5 remained perinuclear and was also associated with the poles of the mitotic spindle (Fig. 3J-L, right cell; Fig. 4D). Upon onset of anaphase, OOC-5 localized on the entire mitotic spindle (Fig. 3D-F,J-L, left cell). The transition from perinuclear OOC-5 localization to general spindle localization coincides precisely with nuclear envelope breakdown in *C. elegans* (Lee et al., 2000). Similar patterns of OOC-5 localization were observed in four-cell embryos, depending on the stage of the cell cycle (Fig. 3M-O, Fig. 4J). In addition, OOC-5 was enriched at the regions of cell-cell contact in some two-cell embryos (40%, *n*=41) and virtually all four-cell embryos examined (96%, *n*=24; Fig. 3M, Fig. 4J).

The perinuclear and reticular nature of OOC-5 coupled with the cell cycle-dependent changes in localization resemble the localization pattern of endoplasmic reticulum (ER) proteins in several organisms (Lippincott-Schwartz et al., 2000; Terasaki, 2000). Additionally, human Torsin A was recently shown to co-localize with the ER resident proteins BiP and PDI when expressed in cultured cells (Hewett et al., 2000; Kustedjo et al., 2000). To test whether OOC-5 associates with the ER in the *C. elegans* embryo, we screened antibodies against conserved ER antigens for cross reactivity with *C. elegans* embryos (see Materials and Methods). The 2E7 antibody, raised against the HDEL C-terminal peptide that serves as a retention signal on resident ER proteins (Munro and Pelham, 1987; Napier et al., 1992), stained *C. elegans* embryos in a pattern consistent with the ER in other systems (Fig. 4). To determine if OOC-5 and HDEL staining co-localize, we double-labeled embryos with antibodies against both OOC-5 and HDEL. OOC-5 co-localized with the HDEL staining in all one to four-cell embryos examined (*n*=144), at all stages of the cell cycle (Fig. 4). These data suggest that OOC-5 protein is localized to the ER or secretory pathway in *C. elegans* embryos.

OOC-5 is mislocalized in *ooc-3* embryos, germlines and intestinal cells

The *ooc-5* mutant phenotype is virtually identical to that of *ooc-3* mutants (Basham and Rose, 1999; Pichler et al., 2000). *ooc-3* was recently cloned and encodes a novel protein containing multiple potential transmembrane regions; OOC-3 protein was also reported to co-localize with HDEL staining (Pichler et al., 2000). Because the OOC-3 and OOC-5 antibodies were made in the same species, double-immunolocalization studies could not be performed directly. Therefore, to provide a comparison to OOC-5 protein distribution, we double-labeled wild-type embryos with antibodies against OOC-3 and HDEL. OOC-3 and HDEL staining co-localized in wild-type one to four-cell embryos (*n*=81) at all cell cycle stages and was indistinguishable from OOC-5/HDEL double labeling (Fig. 4). These results suggest that OOC-5 and OOC-3 localize to the same subcellular structures.

To investigate the interdependency of OOC-5 and OOC-3 localization, we examined OOC-3 and HDEL localization in *ooc-5* mutant embryos and OOC-5 and HDEL localization in *ooc-3* mutant embryos. OOC-3 and HDEL co-localized in all double-labeled *ooc-5* embryos examined (*n*=67). The majority of these embryos had OOC-3 and HDEL staining comparable with wild type (85%; Fig. 5A-I), although in some mutant embryos the reticular staining appeared slightly clumped. We conclude that *ooc-5* function is not essential for normal OOC-3 localization to HDEL-labeled structures.

By contrast, OOC-5 localization was disrupted at all stages in *ooc-3* mutant embryos. In interphase and prophase cells, the OOC-5 reticular staining appeared greatly reduced compared with wild type, while strong perinuclear staining was still observed (Fig. 5J,P). HDEL staining appeared comparable with wild type in most interphase and prophase *ooc-3* embryos (75%, *n*=36; Fig. 5K), but, like OOC-5 staining, appeared less reticular in the remaining embryos (Fig. 5Q). In the majority of metaphase through telophase *ooc-3* embryos, OOC-5 staining on mitotic spindles was greatly diminished (96%, *n*=27; Fig. 5M). In addition, clumps of HDEL staining were seen throughout the cytoplasm and distributed unevenly on mitotic spindles in most metaphase through telophase embryos (70%, *n*=27; Fig. 5N). The reduction in OOC-5 signal intensity in *ooc-3* embryos compared with wild type was seen with multiple preparations, and this reduction was consistently more pronounced than the reduction in HDEL staining. From these data, we conclude that the localization of OOC-5 to HDEL-labeled structures depends upon *ooc-3* at all stages of the cell cycle. In addition, the abnormalities in HDEL staining suggest that *ooc-3* is required for the normal localization of HDEL antigens and/or the normal morphology of HDEL-labeled structures, especially during mitosis.

The conclusion that OOC-5 localization depends upon *ooc-3* function is further supported by observations of OOC-5 localization in *ooc-3* mutant germlines and intestinal cells. The wild-type germline consists of two U-shaped gonads, each containing a distal arm of syncytial nuclei connected to a proximal arm with a single row of oocytes. In the proximal arm of the wild-type germline, OOC-5 and HDEL staining appeared diffuse and no strong nuclear rim staining was observed (Fig. 6A,B). By contrast, both OOC-5 and HDEL staining were enriched at the perinuclear region of *ooc-3* oocytes (Fig. 6E,F). This abnormal pattern of localization is not simply a consequence of the reduced size of *ooc-3* oocytes, as OOC-3 and HDEL localization appears normal in comparably small *ooc-5* oocytes (Fig. 6G,H). Finally, OOC-5 was similarly mislocalized in *ooc-3* mutant intestinal cells. In wild-type intestinal cells, both OOC-5 and HDEL staining are observed throughout the cytoplasm but do not appear enriched around the nuclei (Fig. 6I,J). However, *ooc-3* mutant intestinal cells frequently had strong OOC-5 perinuclear staining (Fig. 6M). These data indicate that OOC-5 localization depends upon the *ooc-3* gene in oocytes and intestinal cells as well as in embryos.

GLP-1 localization is normal in early *ooc* mutant embryos

The co-localization of OOC-5 and OOC-3 with HDEL staining suggests that these proteins function in the secretory pathway. The observation that both OOC-5 and HDEL staining are abnormal in *ooc-3* mutants raises the possibility that the polarity defects observed in *ooc* mutant embryos are due to a nonspecific disruption of secretion. To determine if general secretion is abnormal in *ooc* mutant embryos, we examined embryos for the localization of GLP-1, a known transmembrane protein. Translational control of *glp-1* mRNA results in GLP-1 protein being restricted to anterior blastomeres in early wild-type embryos; GLP-1 localizes to the AB cell of two-cell embryos and the AB daughters (ABa and ABp) at the four-cell stage (Evans et al., 1994). In wild-type embryos stained with GLP-1 antibodies, plasma membrane localization was evident in some two-cell (3/13) and all four-cell embryos (n=13; Fig. 7B) (Evans et al., 1994). In *ooc-5* and *ooc-3* mutant embryos, GLP-1 localization appeared normal, being present only in the AB cell and the AB daughters (n=26 for *ooc-5*; n=20 for *ooc-3*; Fig. 7C,D). In addition, GLP-1 staining at the plasma membrane was comparable with that in wild-type: GLP-1 was detected at the plasma membrane separating AB and P_1 in some two-cell mutant embryos (1/9 for *ooc-5*; 3/9 for *ooc-3*) and around the plasma membrane of both AB daughters in all four-cell embryos (n=17 for *ooc-5*; n=11 for *ooc-3*; Fig. 7D). The normal membrane localization of GLP-1 indicates that the secretory system is still functioning in *ooc-5* and *ooc-3* mutant embryos. Furthermore, these results strengthen our previous findings that anterior-posterior polarity is established normally in one-cell *ooc* mutant embryos (Basham and Rose, 1999), because it has been shown that mutations that disrupt one-cell polarity also disrupt the spatial control of GLP-1 expression (Crittenden et al., 1997). Taken together, these results support a model in which *ooc-5* and *ooc-*

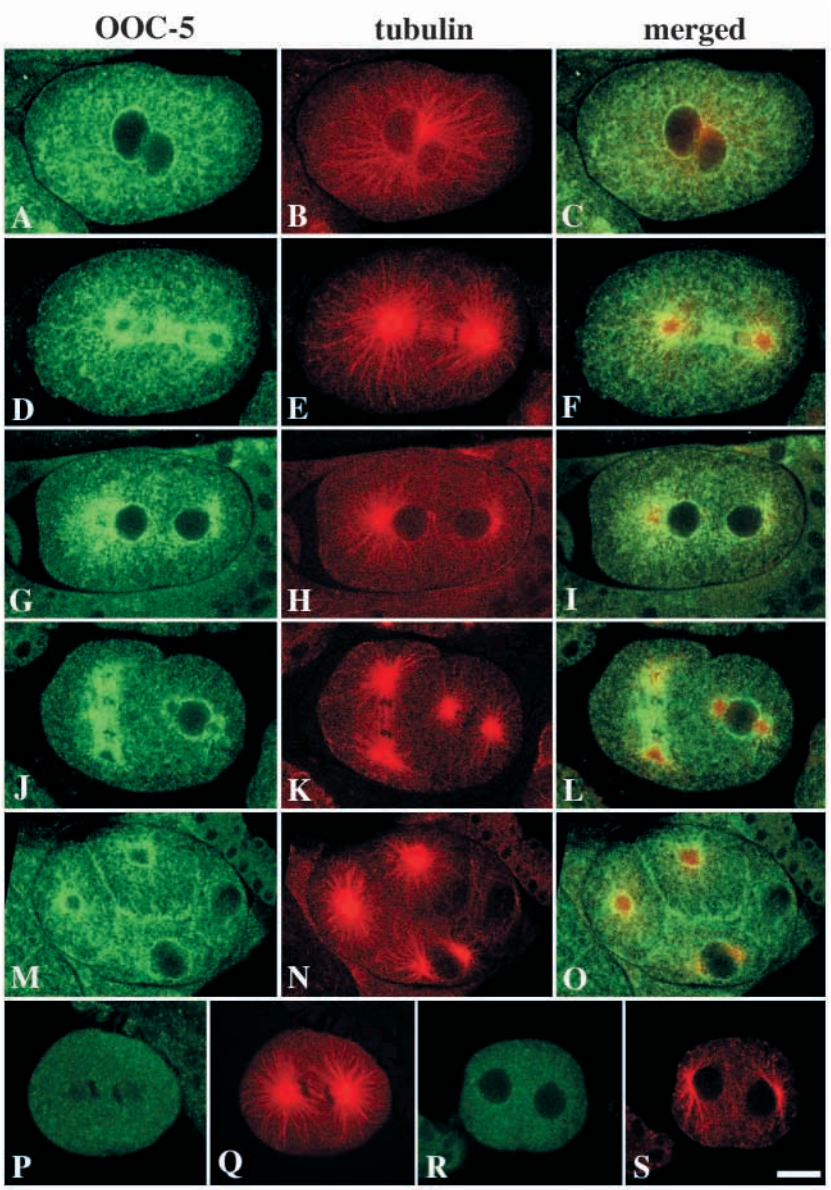

Fig. 3. Distribution of OOC-5 in embryos. Confocal micrographs of wild-type embryos (A-O) double labeled with anti-OOC-5 (A,D,G,J,M) and anti-tubulin antibodies (B,E,H,K,N); merged images are shown in C,F,I,L,O, where the overlap in signal appears yellow. Confocal images of *ooc-5(it145)* mutant embryos double labeled with anti-OOC-5 (P,R) and anti-tubulin (Q,S) antibodies. (A-C) One-cell embryo at prophase; (D-F) one-cell anaphase; (G-I) two-cell interphase; (J-L) two-cell embryo in which the AB cell (left) is in anaphase and P1 is in metaphase. (M-O) Four-cell embryo in which the AB cells are dividing (top left cells, spindle poles only visible), EMS is in prophase and P2 (right most cell) is in interphase. (P-Q) One-cell anaphase *ooc-5* mutant embryo. (R,S) Two-cell interphase *ooc-5* mutant embryo. The stage of the cell cycle was determined by DAPI staining (not shown) in addition to microtubule staining for all embryos. Posterior is towards the right in this and all figures. Scale bar: 10 μm.

3 function in the secretion of a specific subset of proteins required for polarized domains in the P_1 cell.

DISCUSSION

OOC-5 and OOC-3 may function in the secretory pathway

Our immunolocalization results show that OOC-5 localizes to all blastomeres of the early embryo in the same pattern as that seen for ER proteins in other systems. Furthermore, OOC-5 staining is indistinguishable from that of the 2E7 antibody, which was generated against a peptide with HDEL at its C terminus (Napier et al., 1992). The HDEL sequence functions as a C-terminal ER retention signal in several organisms (Monnat et al., 2000; Munro and Pelham, 1987; Pelham, 1990). In plants, the 2E7 antibody recognizes ER resident proteins with a C-terminal HDEL sequence, but does not recognize proteins bearing the KDEL retention signal, indicating that this antibody is highly specific (Napier et al., 1992). Several *C. elegans* proteins predicted to be residents of the ER have a C-terminal HDEL and are the likely targets of the 2E7 antibody. Our results are thus consistent with localization of OOC-5 to the secretory pathway, in particular the endoplasmic reticulum. This view is further strengthened by studies showing that a human homolog of OOC-5, Torsin A, co-localizes with the ER resident enzymes BiP and PDI when overexpressed in cultured cells. In addition, Torsin A fractionated as a lumenal ER protein in extracts from such cells (Hewett et al., 2000; Kustedjo et al., 2000). Taken together, these results strongly suggest that OOC-5 is an ER protein, which implies a molecular link between secretion and the re-establishment of polarized domains in the *C. elegans* embryo.

The OOC-3 protein localizes to the same subcellular structures as OOC-5, and is required for normal OOC-5 localization. Thus, OOC-3 may play a role in some aspect of OOC-5 modification, folding or targeting within the secretory system. With this model, OOC-3 would play a similar role for one or more proteins recognized by the 2E7 antibody. An alternative possibility is that the changes in OOC-5 localization in *ooc-3* mutants are a secondary effect of defects in the morphology of HDEL-labeled structures. The generation of additional ER markers for *C. elegans* embryos will allow us to

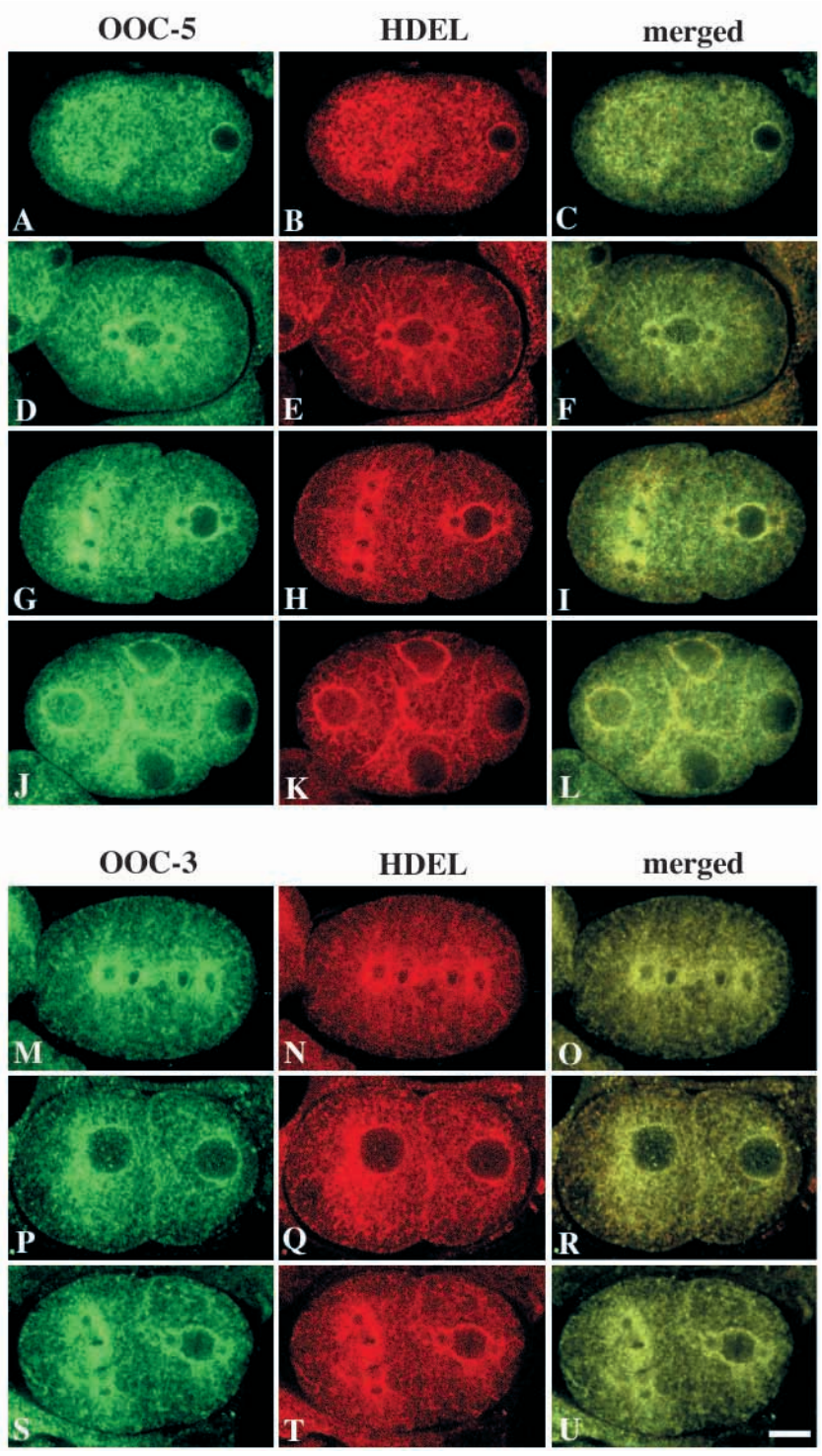

Fig. 4. Comparison of OOC-5, OOC-3 and HDEL staining in wild-type embryos. Confocal micrographs of wild-type embryos double labeled with anti-OOC-5 (A,D,G,J) or anti-OOC-3 (M,P,S) and anti-HDEL antibodies (B,E,H,K,N,Q,T). Merged images showing the extent of signal overlap (yellow) are shown in C,F,I,L,O,R,U. (A-L) Co-localization of OOC-5 and HDEL. (A-C) One-cell prophase embryo with one pronucleus visible; the other pronucleus is out of the plane of focus. (D-F) One-cell embryo in metaphase. (G-I) Two-cell in which AB (left cell) is in anaphase and P1 is in metaphase. (J-L) Four-cell embryo in which the AB cells (top left cells) are in prophase and EMS and P2 are in interphase. (M-U) Co-localization of OOC-3 and HDEL staining. (M-O) One-cell embryo in anaphase. (P-R) Two-cell embryo in early interphase. (S-U) Two-cell embryo in which AB is in anaphase and P1 is in metaphase. The stage of the cell cycle was determined by DAPI staining (not shown) in all cases. Scale bar: 10 μm.

distinguish between changes in overall ER morphology and the mislocalization of specific proteins.

OOC-5 is a Torsin-related protein of the AAA superfamily of ATPases

The sequence of the predicted OOC-5 protein also provides some clues as to possible function. OOC-5 is most highly related to the Torsin family of proteins, which has members in *C. elegans*, *Drosophila*, mouse and human, and OOC-5 is more distantly related to the Clp/Hsp100 family of bacteria and yeast. The Torsins and Clp/Hsp100 related proteins have recently been included as part of the expanded AAA family of ATPases (also referred to as AAA+) (Neuwald et al., 1999; Vale, 2000). Although the cellular roles of AAA family members are quite diverse, a large number of these proteins have chaperone activity; that is, they participate in the remodeling of proteins, either to facilitate the folding of an individual protein or assembly of a complex, or to facilitate unfolding or disassembly. For those AAA members for which structural and biochemical data are available, including the Clp/Hsp 100 members, a common feature is the formation of an oligomeric ring. The hydrolysis of ATP by the subunits of the ring is proposed to produce a conformational change that does the work of assembly or disassembly (Neuwald et al., 1999; Schirmer et al., 1996; Vale, 2000). Thus, it is possible that OOC-5 plays a role as a chaperone in the ER.

A model for OOC-5 and OOC-3 function

Based on these results, we propose that OOC-5 and OOC-3 function in the ER in the secretion of proteins required to set up asymmetric plasma membrane domains required for proper polarity in the P_1 cell at the two-cell stage. Specifically, OOC-3 would assist in the proper modification or localization of OOC-5, which would then act as an ER chaperone for proteins destined for the plasma membrane. What are the substrates for this proposed OOC-5 activity? It seems unlikely that any of the

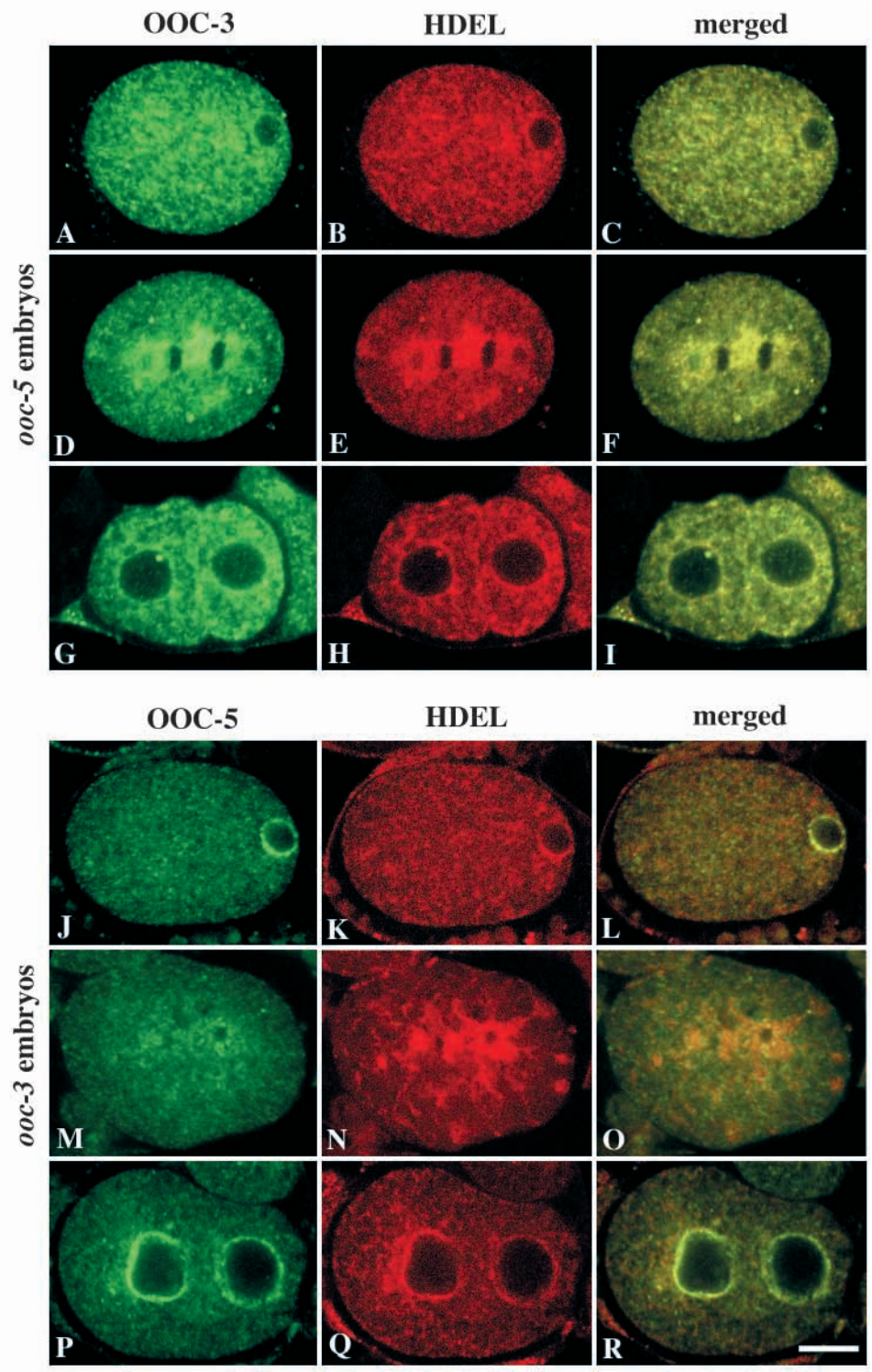

Fig. 5. OOC-5 localization depends on OOC-3 in embryos, but OOC-3 localization does not require OOC-5. (A-I) Confocal micrographs of *ooc-5 (it145)* embryos double labeled with anti-OOC-3 (A,D,G) and anti-HDEL antibodies (B,E,H). Merged images show the extent of co-localization (C,F,I). (A-C) One-cell prophase embryo with one pronucleus visible; the other pronucleus is out of the plane of focus. (D-F) One-cell embryo in anaphase. (G-I) Two-cell embryo in interphase. **(**J-R) Confocal micrographs of *ooc-3 (mn241)* embryos double labeled with anti-OOC-5 (J,M,P) and anti-HDEL antibodies (K,N,Q). Merged images show the extent of co-localization (L,O,R). (J-L) One-cell prophase embryo with one pronucleus visible. (M-O) One-cell embryo in anaphase. (P-R) Two-cell interphase. All confocal images were obtained using the same settings as for the wild-type embryos in Fig. 4. Scale bar: 10 μm.

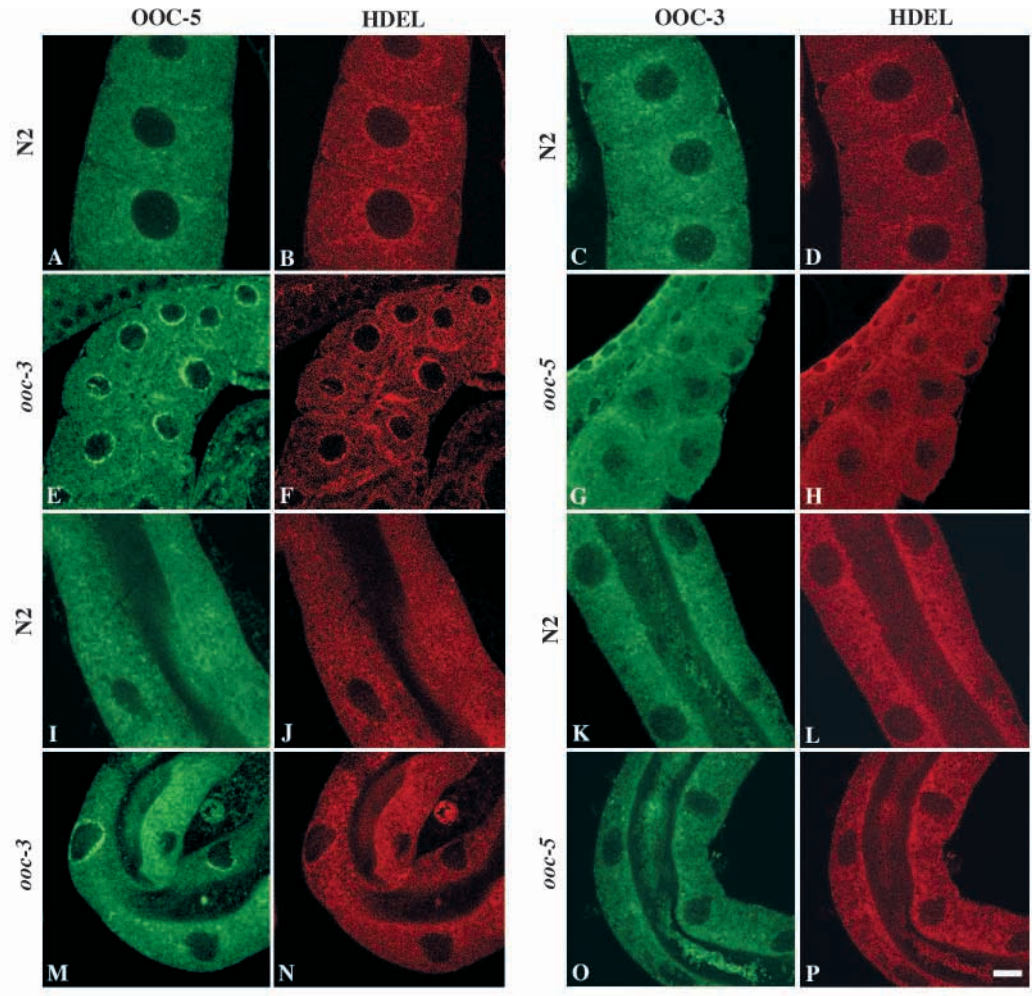

Fig. 6. OOC-5 and OOC-3 localization in germlines and intestinal cells. Confocal micrographs of germlines and intestinal cells double labeled with either anti-OOC-5 (A,E,I,M) or anti-OOC-3 antibodies (C,G,K,O) and anti-HDEL antibodies (B,D,F,H,J,L,N,P). (A-H) Oocytes in proximal germlines of wild type (N2) and mutant worms. (I-P) Intestinal cells. Scale bar: 10 μm.

PAR proteins or the PAR-3/PAR-6/PKC complex are direct targets of OOC-5, because all of these proteins lack predicted ER signal peptides and transmembrane spanning sequences. Biochemical and genetic interaction screens could potentially identify the interconnections between OOC-5, secretion, and PAR localization in the future.

The fact that *ooc-5* and *ooc-3* mutations are pleiotropic, and that HDEL-labeled structures appear abnormal in *ooc-3* mutants could be interpreted to mean that at least OOC-3 is involved in general secretion or ER function, rather than in the specific role presented above. However, the phenotypes of *ooc-3* and *ooc-5* with respect to polarity and spindle orientation are indistinguishable, and several observations argue against a role for these proteins in general secretion. First, the nature of the defects in PAR localization in *ooc-5* and *ooc-3* indicate a specific effect on polarity (Basham and Rose, 1999; Pichler et al., 2000). Second, inhibition of function of the general secretory pathway proteins EMO-1, β′ COP and ARF1 result in complete sterility of worms, and/or production of abnormal polynucleate oocytes with weak egg shells (M. F. Tsou and L. S. R., unpublished) (Grant and Hirsh, 1999; Iwasaki et al., 1996). These phenotypes are distinct from, and much more severe than, the small oocytes with altered polarity observed for *ooc-3* and *ooc-5* mutants. The *ooc* phenotypes described most likely represent the null phenotype; although the other

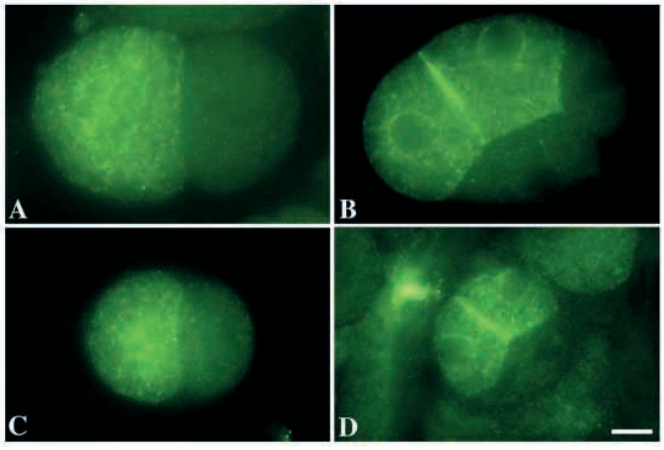

Fig. 7. Distribution of GLP-1 in embryos. Digital images of wild-type (A-B) and *ooc-5* embryos (C-D) labeled with anti-GLP-1 antibodies. (A) Two-cell wild-type embryo with GLP-1 localization in the AB cell. (B) Four-cell wild-type embryo with GLP-1 localization at the plasma membrane and in the cytoplasm of ABa and ABp. (C) Two-cell *ooc-5* embryo with GLP-1 localization in the AB cell. (D) Four-cell *ooc-5* embryo with GLP-1 localization at the plasma membrane and in the cytoplasm of ABa and ABp. Scale bar: 10 μm.

torsin-related proteins of *C. elegans* could be redundant with *ooc-5*, there are no other *C. elegans* proteins with similarity to OOC-3 (Basham and Rose, 1999; Pichler et al., 2000). Finally, GLP-1, a known transmembrane protein, is localized normally in early *ooc* mutant embryos, indicating that general secretion is still functioning in these embryos.

The phenotype of *ooc* mutants also argues for a role in secretion required specifically for polarity at the two-cell stage. In the one-cell embryo, polarity is established in response to a cue provided by the sperm aster, resulting in the establishment of asymmetric PAR protein domains (Goldstein and Hird, 1996; Wallenfang and Seydoux, 2000). Studies of the *pod* genes have implicated membrane trafficking in establishing this initial polarity (Rappleye et al., 1999; Tagawa et al., 2001). *pod* mutant embryos have abnormal eggshells, are osmotically sensitive, lack cytoplasmic flow and have delocalized PAR protein domains at the one-cell stage. The *ooc* mutant embryos do not show these defects and PAR protein domains are localized normally at the one-cell stage, but are mislocalized at the two-cell stage (Basham and Rose, 1999; Pichler et al., 2000). Thus, the *ooc* and *pod* genes appear to act in different pathways for generating asymmetry and suggest that additional mechanisms are required for generating polarized domains at the two-cell stage.

There are several precedents for ER localized proteins that function in the secretion of one or a subset of proteins involved in cell polarity or axial patterning. For example, in *Drosophila*, the *windbeutel* gene encodes an ER protein that is required for the proper localization of Pipe (a homolog of vertebrate glycosaminoglycan-modifying enzymes) to the Golgi. The functions of Windbeutel and Pipe are required to activate ventral patterning of the embryo (Sen et al., 2000; Sen et al., 1998). Likewise, the ER vesicle protein Erv14p is required in the budding yeast *S. cerevisiae* for the secretion of Ax12 protein at bud tips, where Axl2 functions in the axial pattern of bud-site selection. Null mutations in Erv14 result in the accumulation of Ax12 in the ER and loss of axial budding pattern, but no general defect in bud growth (Powers and Barlowe, 1998). Mutations in the *Drosophila* homolog of erv14, *cornichon*, also result in specific axial patterning defects during oogenesis (Roth et al., 1995).

Does OOC-5 function in secretion of specific proteins in other cell types? Although only diffuse staining was observed for OOC-5 and OOC-3 in intestine cells and the germ line, the mislocalization of OOC-5 in both cell types in *ooc-3* mutants was striking. This suggests that both proteins could play a role in these tissues as well. A defect in secretion from the intestine, which secretes the yolk proteins taken up by oocytes (Grant and Hirsh, 1999), could explain the small oocyte size seen in *ooc* mutants. Alternatively, the disorganized germline and reduced oocyte size could result from a defect in secretion in the germline itself.

The *C. elegans* embryo as a model for the study of Torsin-related proteins

The *C. elegans* embryo should serve as a model for investigating at least the basic biochemical function of Torsin proteins, given that OOC-5 and other Torsins share significant sequence identity over their entire lengths. In addition, OOC-5 and Torsin A both co-localize with markers of the endoplasmic reticulum. OOC-5 may also be relevant to early-onset torsion dystonia, the disease caused by mutations in the gene for human Torsin A. Early-onset dystonia appears to be caused by altered neuronal function, and Torsin A is highly expressed in neurons (Hewett et al., 2000; Ozelius et al., 1997; Ozelius et al., 1998). Although at first glance a human neuron bears no resemblance to the P_1 cell of the *C. elegans* embryo, both are polarized cells. Neurons are highly asymmetric, with specialized plasma membrane domains, or synapses that are equipped for signal reception versus signal transmission; polarized secretion delivers neurotransmitters and other proteins to synapses (Bredt, 1998). An intriguing possibility is that wild-type Torsin A is normally involved in cellular polarity and/or polarized secretion, and that the mutant form disrupts these processes, resulting in dystonia. Indeed, studies have shown possible defects in dopamine-mediated transmission in individuals with early-onset dystonia, leading to the idea that the mutant form of Torsin A interferes with membrane trafficking along axons (Hewett et al., 2000; Ozelius et al., 1997; Ozelius et al., 1998). In addition, it has recently been shown that mammalian PAR-3 and PAR-6 are expressed in neurons of the mouse brain, making the potential parallels between the *C. elegans* embryo and neurons even stronger (Lin et al., 2000). Thus, further studies of the biochemical function of OOC-5 and the identification of other proteins that function in the OOC-5 pathway could provide insights for the understanding of both Torsin function and the defects that cause dystonia.

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