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α-Endosulfine is a conserved protein required for oocyte meiotic maturation in *Drosophila*

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Meiosis is coupled to gamete development and must be well regulated to prevent aneuploidy. During meiotic maturation, *Drosophila* oocytes progress from prophase I to metaphase I. The molecular factors controlling meiotic maturation timing, however, are poorly understood. We show that *Drosophila α-endosulfine* (*endos*) plays a key role in this process. *endos* mutant oocytes have a prolonged prophase I and fail to progress to metaphase I. This phenotype is similar to that of mutants of *cdc2* (synonymous with *cdk1*) and of *twine*, the meiotic homolog of *cdc25,* which is required for Cdk1 activation. We found that Twine and Polo kinase levels are reduced in *endos* mutants, and identified Early girl (Elgi), a predicted E3 ubiquitin ligase, as a strong Endos-binding protein. In *elgi* mutant oocytes, the transition into metaphase I occurs prematurely, but Polo and Twine levels are unaffected. These results suggest that Endos controls meiotic maturation by regulating Twine and Polo levels, and, independently, by antagonizing Elgi. Finally, germline-specific expression of the human α-endosulfine ENSA rescues the *endos* mutant meiotic defects and infertility, and α-endosulfine is expressed in mouse oocytes, suggesting potential conservation of its meiotic function.

KEY WORDS: Meiosis, Oogenesis, α-Endosulfine, Cdc25, Polo, E3 ubiquitin ligase, *Drosophila*

INTRODUCTION

Meiosis is a fundamental process required for gamete production. Oocytes undergo two meiotic arrests to accommodate their growth and differentiation (Kishimoto, 2003; Page and Orr-Weaver, 1997; Sagata, 1996; Whitaker, 1996). The prophase I arrest is highly conserved, whereas the second block in metaphase I or II is species specific. The prophase I arrest lasts for prolonged periods, even decades in humans. In response to hormonal and/or developmental cues, this arrest is released and meiosis progresses to metaphase I, a process known as meiotic maturation. The precise timing of meiotic maturation ensures normal chromosome segregation and viable oocytes.

In *Drosophila melanogaster*, each oocyte develops within a germline cyst that includes fifteen nurse cells, and follicle cells surround each cyst to form an egg chamber, which develops through fourteen stages (Spradling, 1993). The oocyte initiates meiosis following cyst formation and remains in prophase I for days (King, 1970). During prophase I, chromosomes become condensed into a spherical karyosome, with a spot of concentrated heterochromatin. Meiotic maturation occurs during stage 13: the nuclear envelope breaks down, chromosomes condense and the meiotic spindle is assembled, culminating in the second meiotic arrest in metaphase I (King, 1970). Metaphase I is marked by a bipolar meiotic spindle, exchange chromosomes positioned at the metaphase plate, and small non-exchange, highly heterochromatic fourth chromosomes localized between the metaphase plate and the poles (Theurkauf and Hawley, 1992). Mature stage 14 oocytes remain in metaphase I and dehydrate. In the oviduct, water re-absorption is thought to promote the completion of meiosis (Mahowald et al., 1983).

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In several systems, high activity of the serine/threonine kinase Cdk1 (also known as Cdc2) and its regulatory subunit Cyclin B are required for meiotic maturation (Kishimoto, 2003; Sagata, 1996). Cdk1 activity is stimulated by the Cdc25 phosphatase, which removes inhibitory phosphates on Cdk1. Cyclin B/Cdk1 activity is low during prophase I, while an activity increase triggers meiotic maturation via the phosphorylation of factors involved in nuclear envelope breakdown, chromosome condensation and spindle assembly (Kishimoto, 2003). Although much less is known about *Drosophila* meiotic maturation, in mutants of *twine*, the germline-specific *cdc25* homolog, oocytes do not progress to a normal metaphase I (Alphey et al., 1992; Courtot et al., 1992; White-Cooper et al., 1993), suggesting that high Cdk1 activity is likewise required here. In addition, Cyclin B dynamically associates with the meiotic spindle in *Drosophila*, indicating a potential role in spindle organization (Swan and Schupbach, 2007).

Multiple mechanisms ensure low Cdk1 activity during prophase I in *Drosophila*. The anaphase-promoting complex/cyclosome (APC/C) induces cyclin degradation (Vodermaier, 2004). In female-sterile mutants of *morula*, which encodes the APC/C subunit APC2, Cyclin B accumulates in germline cysts, leading to nurse cell arrest in a metaphase-like state (Kashevsky et al., 2002; Reed and Orr-Weaver, 1997). Cyclin translational repression by Bruno, encoded by *arrest*, also contributes to low Cdk1 activity during prophase I, and *arrest* mutant cysts accumulate Cyclin A and B (Sugimura and Lilly, 2006). High levels of Dacapo, a Cdk1 inhibitor, within the oocyte are likely to contribute to the prophase I arrest (Hong et al., 2003). It is much less understood how these repressive mechanisms are alleviated or how meiotic maturation timing is precisely controlled.

α-Endosulfines are small phosphoproteins of largely unknown functions. Studies of mammalian α-endosulfines in culture suggested a possible role in insulin secretion (Bataille et al., 1999); however, this has not been demonstrated in vivo. Moreover, the expression of α-endosulfines in many tissues (Heron et al., 1998) suggests that they play multiple roles. Our previous studies showed that *Drosophila α-endosulfine* (*endos*) is required for normal

oogenesis rates, stage 14 oocyte dehydration, and fertility (Drummond-Barbosa and Spradling, 2004). Here, we demonstrate for the first time in any system that *endos* is required for meiotic maturation. *endos* mutant oocytes have delayed nuclear envelope breakdown and fail to progress into metaphase I. This defect is remarkably similar to that of *twine* and *cdc2* mutants, and *endos* mutants have reduced expression of Twine and Polo kinase, another cell cycle regulator. In an in vitro binding screen, we identified Early girl (Elgi), a predicted E3 ubiquitin ligase, as a strong Endos interactor. *elgi* disruption resulted in premature transition from prophase I to metaphase I, although it did not rescue Twine or Polo levels in *endos* mutants. We propose that Endos promotes expression of Polo and Twine post-transcriptionally, and has a separate role through the inhibition of Elgi to promote meiotic maturation. Remarkably, germline-specific expression of ENSA, the human α-endosulfine, rescues the *endos* mutant meiotic defect, and α-endosulfine is expressed in mouse oocytes; these data suggest that the meiotic function of α-endosulfine is conserved.

MATERIALS AND METHODS

Drosophila strains and culture

Fly stocks were maintained at 22-25°C on standard medium. *yw* was a control. $endos^{00003}$, $endos^{EY01105}$, twe^{1}, $cdc2^{E1-24}$ and $cdc2^{B47}$ alleles have been described (Alphey et al., 1992; Courtot et al., 1992; Drummond-Barbosa and Spradling, 2004; Stern et al., 1993; White-Cooper et al., 1993). $cdc2^{E1-24}/cdc2^{B47}$ females raised at 18°C were analyzed after incubation at 29°C for 1-2 days. The *P*-element insertion *EY10782*, 396 bp upstream of the *elgi* coding sequence, was mobilized to generate deletions. The $elgi^{1}$ deletion removes the first two exons, and $elgi^{2}$ removes 54 base pairs of the coding region (see Fig. 4D). *twe::lacZ*, *UASp-polo*, *nanos-Gal4::VP16* and *tGPH* have been described (Britton et al., 2002; Edgar and O'Farrell, 1990; Santel et al., 1998; Van Doren et al., 1998; Xiang et al., 2007). *twe::lacZ* is a genomic construct modified to express a Twe::β-galactosidase (Twe-β-gal) protein fusion (White-Cooper et al., 1998). Other genetic elements are described in FlyBase (http://flybase.bio.indiana.edu).

To assess $endos^{00003}$ egg fertilization, we used *dj-GFP* males (Santel et al., 1997). All eggs were fertilized, as detected by the presence of GFP-positive sperm. Oocyte dehydration was analyzed as described (Drummond-Barbosa and Spradling, 2004).

Transgenic line generation

The *endos* coding region plus 21 base pairs immediately upstream were subcloned into *UASpI* (modified from *pUASp*, T. Murphy, NCBI) to create *pUASp-endos*. Similarly, the ENSA coding region plus the same upstream 21 base pairs from *endos* were used to generate *pUASp-ENSA*. The *twine* coding region was subcloned into *pCS2* (a modified *UASp* vector; E. Lee, Vanderbilt University Medical Center) in frame to a c-Myc tag to generate *UASp-myc::twe*. For *hs-twe*, the *twine* cDNA was subcloned into *pCasper-hs*. Transgenic lines were generated as described (Spradling and Rubin, 1982).

RNA and protein analysis

For western analysis, ovaries or egg chambers were homogenized, electrophoresed and transferred to membranes as described (Drummond-Barbosa and Spradling, 2004). Membranes were blocked with Odyssey Blocking Reagent (LI-COR Biosciences) and probed with 1:100 rabbit polyclonal anti-β-galactosidase (Cappel), 1:50 mouse monoclonal anti-Actin (JLA20, Developmental Studies Hybridoma Bank), 1:10 mouse monoclonal α-Cyclin B (F2F4, Developmental Studies Hybridoma Bank), 1:1000 rabbit polyclonal α-Endos (c302) (Drummond-Barbosa and Spradling, 2004), 1:80 mouse monoclonal anti-Polo (MA294) (Logarinho and Sunkel, 1998), 1:500 MPM2 mouse monoclonal (Upstate), 1:1000 mouse monoclonal anti-c-Myc (9E10, Sigma), or 1:1000 mouse monoclonal anti-Cdk1 (anti-PSTAIR, Sigma) antibodies. Alexa 680-conjugated goat anti-rabbit and anti-mouse (Molecular Probes) secondary antibodies were used at 1:5000 dilution. The Odyssey Infrared Imaging System (LI-COR Biosciences) was used for detection.

Ovarian RNA extracted using TRIzol Reagent (Invitrogen) was reverse transcribed (RT) using Oligo(dT)16 (Applied Biosystems) for priming and SuperScript II reverse transcriptase (Invitrogen). PCR was performed using undiluted or diluted (1:5 and 1:25) RT reactions.

Immunoprecipitation/kinase assay

Immunoprecipitation/Cdk1 kinase assays were performed as described (Gawlinski et al., 2007), using extracts from 200 homogenized stage 14 oocytes per sample. Briefly, following incubation of 10 μl of extract with 0.5 μl of anti-Cdk1 (Gawlinski et al., 2007) or 2.5 μl of anti-Cyclin B antibodies, immunocomplexes were isolated using protein A- or protein G-sepharose beads. Washed beads were incubated with 16 μl of kinase buffer, 3 μM of histone H1.2 and 2 μCi [^{32}P]ATP for 10-20 minutes at 25°C. Detection and quantification were performed using a Typhoon 9200 Imager and ImageQuant 5.2. Parallel immunoprecipitations using 100 μl of extract and 5 μl of rabbit polyclonal anti-Cdk1 or 25 μl of anti-Cyclin B antibodies were subjected to western blotting and quantified using Image J. Average relative intensities of [^{32}P]histone H1 were determined after normalization for immunoprecipitated protein amounts, with control levels arbitrarily set at 1.00.

Immunostaining and microscopy

For oocyte DNA analyses, ovaries were dissected in Grace's insect medium (Life Technologies) and fixed as described. Samples were incubated in 0.5 μg/ml DAPI for 10 minutes, mounted in Vectashield (Vector Laboratories), and analyzed using a Zeiss Axioplan 2. Egg chamber developmental stages were identified as described (Spradling, 1993), but we further subdivided stage 13 as early (11-13 nurse cell nuclei), mid (6-10 nurse cell nuclei), and late (1-5 nurse cell nuclei). Stage 13 and 14 oocyte nuclear envelopes were visualized by differential interference contrast and epifluorescence microscopy. Results were subjected to χ^2 testing.

For visualization of microtubules, ovaries were dissected in Robb's media and fixed in 2× oocyte fix buffer as described (Theurkauf and Hawley, 1992). Stage 14 oocytes were hand dechorionated using dissecting needles (Precision Glide, size 27G 11/4), extracted in 1% PBT and stained with anti-α-tubulin FITC-conjugated antibody (DM1A clone, Sigma) at 1:200 dilution. Washed samples were treated with RNAse A and stained with propidium iodide. For visualization of DNA and spindles during embryonic mitoses, 0- to 3-hour embryos were collected, dechorionated, and shaken vigorously for 2 minutes in 1:1 heptane:methanol. After three washes in methanol, samples were fixed overnight at 4°C in 1 ml of methanol, blocked in PBT plus 5% normal goat serum and 5% bovine serum albumin, stained with anti-α-tubulin-FITC antibody, and analyzed using a Zeiss LSM 510 confocal microscope.

For X-gal staining, ovaries were dissected in Grace's insect medium, fixed, and stained at 37°C for 20-30 minutes as described (Margolis and Spradling, 1995). Samples were mounted and analyzed using a Zeiss Axioplan 2 microscope.

Live imaging

Ovaries were dissected in halocarbon oil 700 (Sigma). Stage 13 egg chambers were injected with 1:20 OliGreen dye (Invitrogen) and 2 mg/ml rhodamine-labeled tubulin (Cytoskeleton). Images were obtained at 20-second intervals using a Leica TCS SP5 inverted confocal microscope and assembled using Image J. Nuclear envelope breakdown duration was measured as the elapsed time from the beginning of nuclear envelope ruffling until entry of tubulin into the nucleus.

Drosophila in vitro expression cloning (DIVEC) binding screen

For the DIVEC binding screen, we used the first release of the *Drosophila* Gene Collection as described (Lee et al., 2005). Briefly, 24-cDNA pools were converted into radiolabeled protein pools. Recombinant glutathione S-transferase (GST)-Endos fusion protein beads were incubated with 1.5 μl of each pool in Buffer A [50 mM Tris (pH 8.0), 200 mM NaCl, 0.1% Tween-20, 1 mM PMSF, 1 mM DTT, 10 μg/ml protease inhibitor cocktail tablets, EDTA free (Roche)] at 4°C for 2 hours, and washed three times with 2.5 ml of Buffer A and once with 2.5 ml of Buffer B [50 mM Tris (pH 8.0), 50 mM NaCl, 1 mM PMSF] in Wizard minicolumns (Promega). Bound proteins eluted in 95°C pre-heated 2× sample buffer were electrophoresed and

detected by autoradiography. Five pools with potential Endos-interacting proteins were subjected to secondary (four cDNAs per pool) and tertiary (single cDNAs) screens.

Mouse tissue analysis

All mice in the present investigation were housed and used in accordance with the National Institutes of Health and institutional guidelines. Immunohistochemistry of 5 μm-thick Bouin's-fixed paraffin-embedded ovarian sections was performed as described (Tan et al., 1999), using rabbit pre-immune or anti-Endos c302 serum at 1:1000 dilution.

RESULTS

endos is required for meiotic maturation

Although mammalian *α-endosulfines* have been proposed to regulate insulin secretion (Bataille et al., 1999), our evidence suggests that *endos* does not control the insulin pathway in *Drosophila* (see Fig. S1 in the supplementary material). Nevertheless, Endos is strongly expressed in the germline, and *endos*^{00003} females are completely sterile and their stage 14 oocytes fail to dehydrate (Drummond-Barbosa and Spradling, 2004). We investigated whether meiosis was affected in *endos*^{00003} females by visualizing the oocyte DNA morphology with 4′,6-diamidino-2-phenylindole (DAPI) (Fig. 1, see Table S1 in the supplementary material). As previously described (King, 1970), wild-type oocytes are in prophase I until stage 12 (Fig. 1B-F,V). In early stage 13, only 5.6% of oocytes had progressed to metaphase I, whereas in mid stage 13, that percentage had increased to 21%; by late stage 13, virtually all oocytes were in metaphase I, and this arrest was maintained in mature stage 14 oocytes. *endos* mutant oocytes arrested in prophase I, as indicated by the typical nuclear morphology; however, this arrest lasted longer, with 90% of the mid stage 13 and 58% of late stage 13 oocytes still being in prophase I (Fig. 1G-K,V). By stage 14, *endos*^{00003} oocytes had exited prophase I, but only 3% were in metaphase I. Instead of progressing into metaphase I, 93% of the oocytes displayed dispersed or visually undetectable DNA. Heteroallelic *endos*^{00003}/*endos*^{EY1105} and hemizygous *endos*^{00003}/*Df(3L)ED4536* females showed similar phenotypes (see Table S1 in the supplementary material). Endos protein is normally expressed throughout the cytoplasm of ovarian germ cells (Drummond-Barbosa and Spradling, 2004), including stage 14 oocytes (J.R.V.S. and D.D.-B., unpublished). Moreover, germline-specific expression of Endos rescued meiotic maturation and fertility in *endos*^{00003} females (see Fig. 5C,E,F). These results indicate that *endos* is required in the germline for oocyte progression from prophase I to metaphase I.

Interestingly, the meiotic defects of *endos* mutant females are reminiscent of defects previously reported for mutants of *twine*, the meiotic *cdc25* homolog (Alphey et al., 1992; Courtot et al., 1992; White-Cooper et al., 1993; Xiang et al., 2007). We examined *twine*^{1} mutant females and found that 4.3% of *twine*^{1} late stage 13 oocytes showed metaphase I arrest, with 84% being still in prophase I (Fig. 1L-P,V). At stage 14, 83% of *twine*^{1} oocytes showed abnormalities similar to those of *endos* mutants. Hemizygous *twine*^{1}/*Df(2L)RA5* females had similar defects (see Table S1 in the supplementary material). In addition, by using a temperature-sensitive *cdc2* mutant

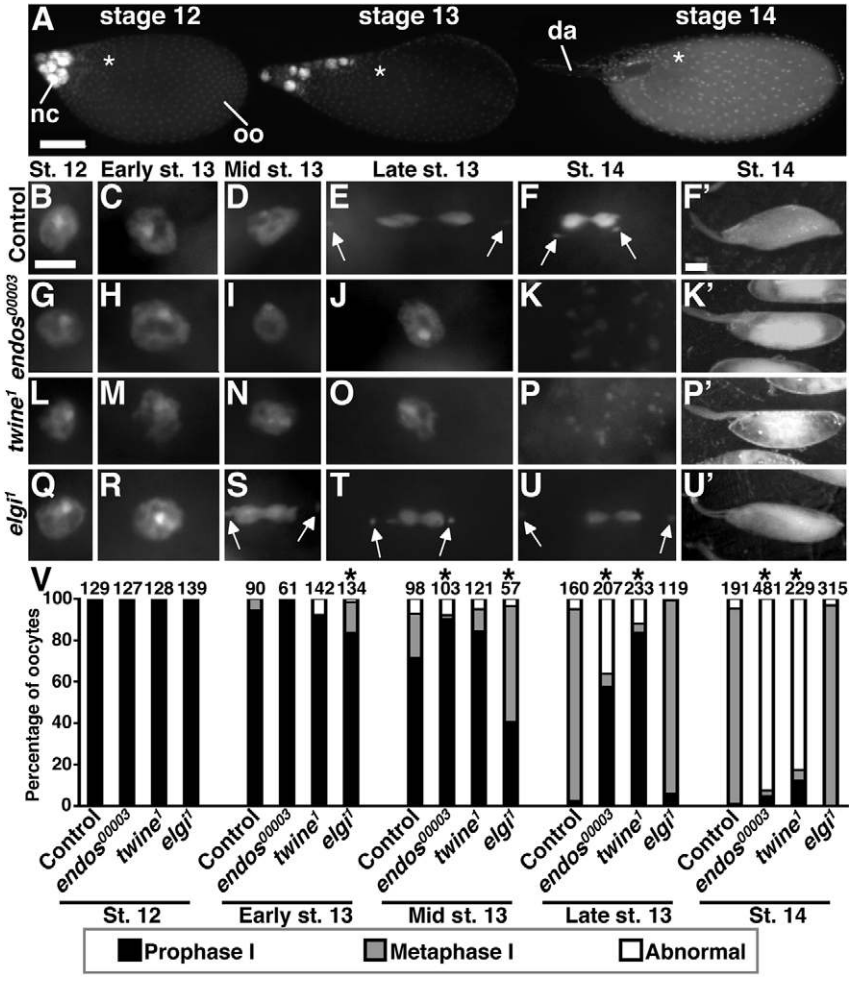

Fig. 1. *endos*^{00003} oocytes fail to undergo meiotic maturation. (**A**) DAPI-stained egg chambers in different stages. nc, nurse cells; oo, oocyte; da, dorsal appendages. Asterisks indicate position of the oocyte nucleus. Scale bar: 100 μm. (**B-F**) Control oocytes in prophase I (B-D) and in metaphase I (E,F). Arrows indicate non-exchange fourth chromosomes. (**G-K**) *endos*^{00003} oocytes have a prolonged prophase I (G-J) and abnormal DNA morphology at stage 14 (K). (**L-P**) *twine*^{1} oocytes show similar phenotypes to *endos*^{00003} oocytes. (**Q-U**) *elgi*^{1} oocytes in prophase I (Q,R) and in premature metaphase I (S-U). Scale bar: 5 μm. Control (**F′**) and *elgi*^{1} (**U′**) have dehydrated stage 14 oocytes. *endos*^{00003} (**K′**) and *twine*^{1} (**P′**) oocytes are not dehydrated and have abnormal yolk morphology. Scale bar: 100 μm. (**V**) Quantification of DNA morphology. The number of oocytes analyzed is shown above the bars. *P<0.001.

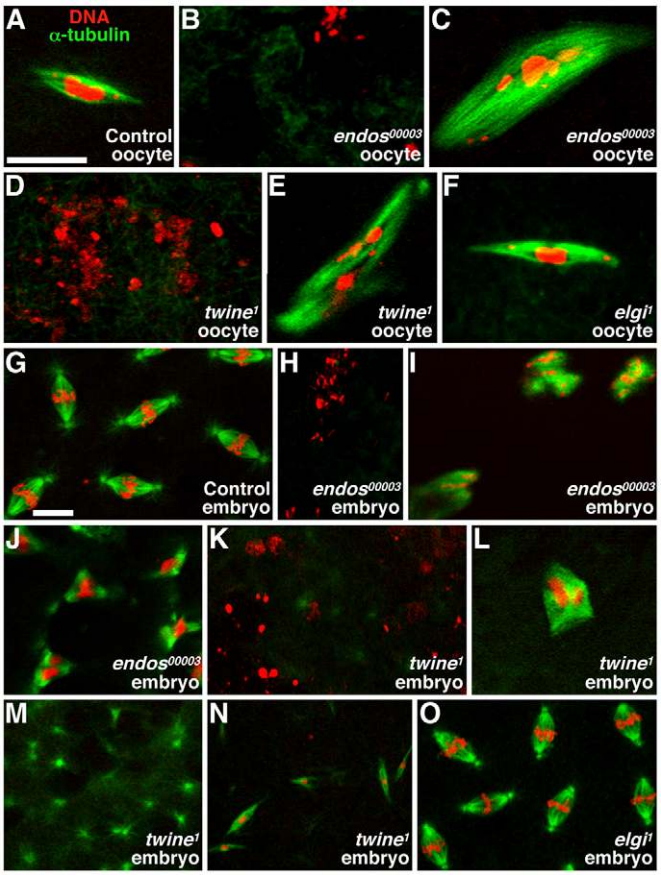

Fig. 2. *endos*^{00003} mutants have spindle defects. Stage 14 oocytes (A-F) and 0- to 3-hour embryos (G-O) stained with propidium iodide (DNA, red) and anti-α-tubulin (microtubules, green). (**A**) Control oocytes in metaphase I. (**B**) Typical *endos*^{00003} oocyte showing dispersed DNA without a spindle. (**C**) Rare *endos*^{00003} oocyte showing abnormal DNA associated with large spindle masses. (**D**,**E**) Similar *twine*^{1} phenotypes. (**F**) *elgi*^{1} oocyte in metaphase I. (**G**) Control embryos exhibiting typical embryonic mitoses. (**H-J**) *endos*^{00003}-derived embryos showing dispersed DNA with no spindle (H), dispersed DNA with spindle masses (I), and tripolar spindles (J). *twine*^{1}-derived embryos showing dispersed DNA (**K**), abnormal spindle masses (**L**), spindle asters with no DNA (**M**), and long, thin spindles (**N**). (**O**) Embryos derived from *elgi*^{1} females showing normal spindles and DNA. Scale bars: 10 μm.

genotype, we also found direct evidence that Cdk1 activity is required for meiotic maturation in *Drosophila*. At the restrictive temperature (29°C), *cdc2*$^{E1\text{-}24}$/*cdc2*^{B47} oocytes showed prolonged prophase I at stage 13 and abnormal DNA morphology at stage 14 (Fig. S2 and Table S1 in the supplementary material). Unfortunately, we could not examine the role of Cyclin B in meiotic maturation because it is required for an earlier role in oogenesis (Wang and Lin, 2005). The similarity between the *endos*^{00003}, *twine*^{1} and *cdc2*$^{E1\text{-}24}$/*cdc2*^{B47} meiotic defects suggests that *endos* may regulate the progression from prophase I to metaphase I through the regulation of *twine* to control Cdk1 activity.

Nuclear envelope breakdown is delayed in *endos* and *twine* mutant oocytes

We next investigated whether *endos* mutants have defects in nuclear envelope breakdown, an expected consequence of insufficient Cdk1 activity (Kishimoto, 2003). We used Nomarski optics for nuclear envelope visualization (see Fig. S3 in the supplementary material). As expected, wild-type oocytes had intact nuclear envelopes in prophase I, whereas those in metaphase I did not. Consistent with the prolonged prophase I of *twine*^{1} and *endos*^{00003} oocytes, the nuclear envelope persisted longer in these mutants. However, although this process was delayed, the nuclear envelope eventually disassembled. These results are consistent with the delayed nuclear envelope breakdown previously reported for *twine*^{1} mutants (Xiang et al., 2007). To extend our analyses, we performed live imaging, measuring nuclear envelope breakdown duration as the elapsed time from the onset of nuclear envelope ruffling until entry of tubulin into the nucleus (see Fig. S4 in the supplementary material). In wild-type oocytes, the nuclear envelope disassembled in ~9 minutes (n=3; measured times were 16, 4 and 6 minutes). By contrast, the nuclear membrane disassembled unevenly and more slowly in *endos*^{00003} (~72 minutes; n=3; measured times were 40, 65 and 111 minutes) and *twine*^{1} (~65 minutes; n=3; measured times were 45, 86 and 63 minutes) oocytes. These findings underscore the similarities between *endos* and *twine* mutants, and are consistent with reduced Cdk1 activity.

Meiotic spindle formation is abnormal in *endos* and *twine* mutant oocytes

High Cdk1 activity induces meiotic spindle formation (Kishimoto, 2003). We thus labeled stage 14 oocytes with anti-α-tubulin fluorescein isothiocyanate (FITC)-conjugated antibodies and propidium iodide to visualize microtubules and DNA, respectively (Fig. 2A-F). While 92% (n=25) of control mature oocytes have the typical metaphase I elongated bipolar spindle (Fig. 2A), this was rarely the case in *endos* or *twine* mutants. Instead, 87% (n=31) of *endos*^{00003} mutants failed to form or maintain the meiotic spindle at stage 14 (Fig. 2B) and a small fraction (13%, n=31) had abnormal spindle-like structures attached to the dispersed DNA (Fig. 2C). Most of the *twine* mutant stage 14 oocytes (82%, n=22) also did not have a meiotic spindle (Fig. 2D); only 7.7% showed normal spindle formation, whereas 18% showed abnormal spindle masses with DNA attached (Fig. 2E). Live imaging indicated that the spindle either failed to form or failed to be maintained in *endos*^{00003} and *twine*^{1} oocytes that ultimately lack spindles (J.R.V.S. and D.D.-B., unpublished). These results support the model that *endos* oocytes have low Cdk1 activity, affecting spindle formation and maintenance.

Maternal *endos* is required for syncytial embryonic mitoses

We reasoned that if *endos* controls meiotic Cdk1 activity, it might have a similar role during early embryonic mitoses. We therefore looked at spindle formation in 0- to 3-hour embryos derived from *endos*^{00003} and *twine*^{1} females (Fig. 2G-O). The majority of wild-type embryos (95%, n=95) showed normal mitotic spindles (Fig. 2G). By contrast, 98% (n=51) of *endos*^{00003}-derived embryos had dispersed (Fig. 2H) or undetectable DNA, resembling the stage 14 oocyte defect. Of those, about 25% had abnormal spindles associated with DNA masses (Fig. 2I). Approximately 2% of *endos*^{00003}-derived embryos appeared to initiate mitotic divisions, but displayed abnormal bipolar, tripolar or multipolar spindles (Fig. 2J). In accordance with a previous report (White-Cooper et al., 1993), the majority of *twine*^{1}-derived embryos (96%, n=74) also showed dispersed (Fig. 2K) or undetectable DNA. Half of those had abnormal spindles associated with DNA masses (Fig. 2L), whereas 8% had free spindle asters (Fig. 2M) and/or thin long spindles (Fig.

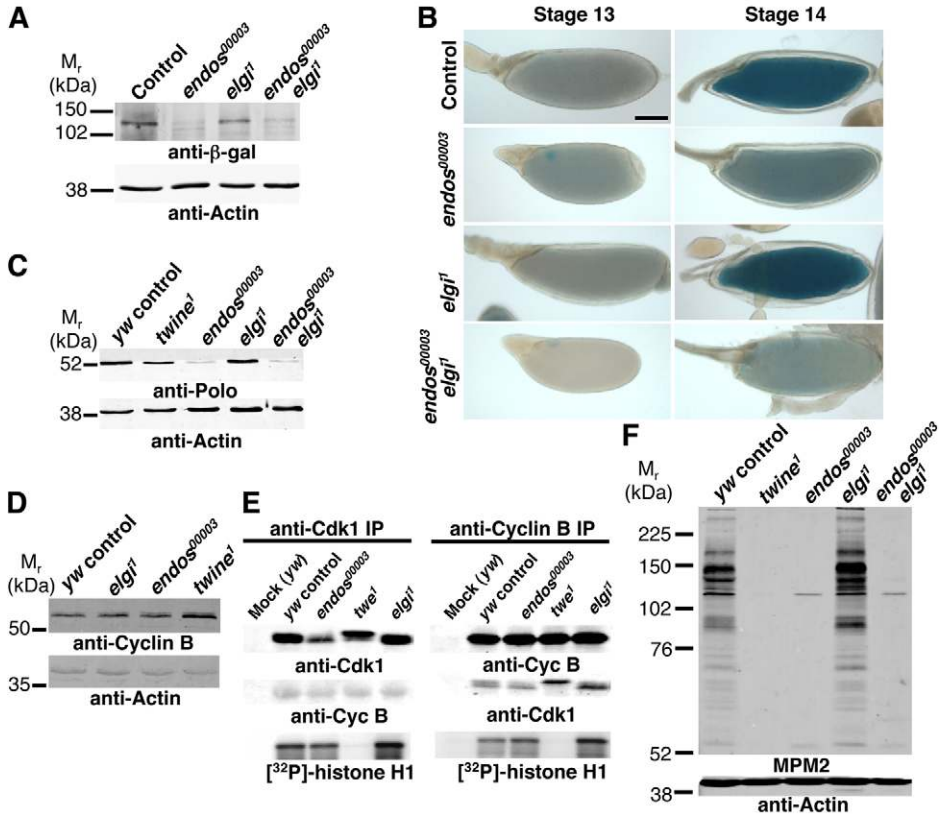

Fig. 3. Endos regulates Twine, Polo and MPM2 phosphoepitopes. (**A**) Anti-β-gal western blot, showing reduced expression of Twine::β-gal in *endos*^{00003} and *endos*^{00003} *elgi*^{1} stage 14 oocytes (30 stage 14 oocytes per lane). (**B**) X-gal staining (blue) of control, *endos*^{00003}, *elgi*^{1} and *endos*^{00003} *elgi*^{1} oocytes reflecting Twine::β-gal levels at stages 13 and 14. Scale bar: 100 μm. (**C**) Polo western analysis of control, *twine*^{1}, *endos*^{00003}, *elgi*^{1} and *endos*^{00003} *elgi*^{1} stage 14 oocytes showing a strong reduction of Polo levels in *endos*^{00003} and *endos*^{00003} *elgi*^{1} (50 stage 14 oocytes per lane). (**D**) Cyclin B western analysis showing normal expression in *endos*^{00003} and *elgi*^{1} mutants, and slightly elevated expression in *twine*^{1} mutants (30 stage 14 oocytes per lane). (**E**) In vitro Cdk1 kinase assay using anti-Cdk1 or anti-Cyclin B immunoprecipitates (IP) from control, *twine*^{1}, *endos*^{00003} and *elgi*^{1} stage 14 extracts. Mock immunoprecipitates were performed without antibodies. Immunoprecipitates were either immunoblotted using anti-Cdk1 or anti-Cyclin B antibodies, or subjected to a kinase assay using [^{32}P]ATP and histone H1 as substrate. (**F**) Western blotting using MPM2 antibodies. Wild-type and *elgi*^{1} oocytes show high MPM2 levels, whereas *endos*^{00003}, *twine*^{1} and *endos*^{00003} *elgi*^{1} oocytes have drastically reduced MPM2 phosphoepitopes (50 stage 14 oocytes per lane). Actin was used as a control.

2N). These results indicate that early embryonic mitoses are also affected in the small percentage of *endos* mutants that initiate those divisions.

endos controls Twine protein levels

In addition to the similar meiotic defects of *dendos*^{00003}, *twine*^{1} and *cdc2*$^{E1\text{-}24}$/*cdc2*^{B47} oocytes, we found that they also share the oocyte dehydration defect observed at stage 14 (Fig. 1F′,K′,P′; see also Fig. S2H′,L′ in the supplementary material). To address whether *endos* might regulate *twine*, we examined the levels of a functional Twine::β-galactosidase (Twine::β-gal) fusion protein (Maines and Wasserman, 1999; White-Cooper et al., 1998) in *endos* mutants. In control and *endos*^{00003} ovarioles, Twine::β-gal expression was low or undetectable until stage 12, and detectable at stage 13. At stage 14, however, Twine::β-gal expression was stronger in control but greatly reduced in *endos*^{00003} oocytes (Fig. 3A,B), despite normal *twine* mRNA levels (J.R.V.S. and D.D.-B., unpublished), suggesting that *endos* is required for Twine upregulation at the post-transcriptional level.

We next tested the ability of heat-shock- or Gal4-inducible *twine* transgenes (*hs-twine* and *UASp-myc::twine*, respectively) to rescue the *endos*^{00003} defects. Robust expression of Myc-tagged Twine was induced by the germline-specific *nanos-Gal4::VP16* driver in control females, and both *twine* transgenes rescued the meiotic defects and sterility of *twine*^{1} females. By contrast, the expression of Myc::Twine was severely reduced in *endos*^{00003} females, and these low Myc::Twine levels did not rescue the *endos*^{00003} defects (see Fig. S5A,B in the supplementary material; D.D.-B. and J.R.V.S., unpublished). The *endos*^{00003} phenotype was similarly not rescued by the *hs-twine* transgene (D.D.-B. and J.R.V.S., unpublished). These data suggest that Endos affects Twine protein stability, although we cannot definitively conclude that this causes the *endos* meiotic defects.

Endos regulates Polo kinase levels independently of Twine

The *Xenopus* polo-like kinase Plx1 phosphorylates and activates Cdc25, leading to Cyclin B/Cdk1 activation (Kumagai and Dunphy, 1996; Qian et al., 2001). In mice, Cyclin B/Cdk1-mediated phosphorylation stabilizes Cdc25A and Cdc25B, creating a positive-feedback loop (Mailand et al., 2002; Nilsson and Hoffmann, 2000). We therefore sought to determine whether Polo kinase was affected in *endos* mutants, potentially explaining their low Twine levels. Strikingly, Polo kinase expression was markedly reduced in

endos^{00003} ovaries and mildly reduced in *twine*^{1} ovaries (Fig. 3C; see also Fig. S6 in the supplementary material), although *polo* mRNA expression was unaffected in *endos*^{00003} oocytes (D.D.-B. and J.R.V.S., unpublished), indicating a post-transcriptional effect. The reduced levels of Polo kinase and Twine in *endos* mutants are not due to a generalized effect on protein expression, as no decrease in Cyclin B was observed (Fig. 3D), but it is conceivable that Twine is unstable as a consequence of reduced Polo levels. Although germline induction of a functional *UAS-polo* (Xiang et al., 2007) increased Polo expression in control ovaries, Polo levels remained very low in *endos*^{00003} oocytes and *endos* defects were not rescued (see Fig. S5C,D in the supplementary material). Thus, we could not determine whether Polo expression is sufficient to rescue the *endos* defects.

endos^{00003} oocytes show normal in vitro Cdk1 kinase activity but reduced in vivo MPM2 phosphoepitopes

The low Polo and Twine levels might lead to reduced Cdk1 activity in *endos*^{00003} oocytes. To address this question, we first performed immunoprecipitation/Cdk1 kinase assays. In control stage 14 oocytes, anti-Cdk1 immunoprecipitates contained Cdk1 and associated Cyclin B, and they phosphorylated histone H1 in vitro [arbitrary relative intensity (R.I.)=1.00, n=9; Fig. 3E]. Similar results were obtained with anti-Cyclin B immunoprecipitates, suggesting that stage 14 oocytes contain active Cyclin B/Cdk1 complexes. *twine*^{1} immunoprecipitates had markedly reduced kinase activity (R.I.=0.13±0.11; n=8; P<0.001). By contrast, Cdk1 in *endos*^{00003} and *elgi*^{1} immunoprecipitates had normal kinase activity (1.52±0.96, n=9, and 1.47±1.02, n=8, respectively; P>0.05 for both), although Cdk1 was present at slightly reduced levels and had altered electrophoretic mobility (it appeared to be hypophosphorylated) in *endos*^{00003} mutants (Fig. 3E; see also Fig. S2M in the supplementary material). These in vitro results suggest that *endos* may not be required for normal Cdk1 activity in maturing oocytes; however, it is equally likely that in vitro phosphorylation of histone H1 may not reflect the endogenous phosphorylation of key substrates in vivo (see Discussion).

MPM2 antibodies recognize conserved phosphoepitopes of mitotic proteins (Davis et al., 1983), and many of the MPM2 epitopes result from Cdk1 activation in vertebrates (Skoufias et al., 2007). In *Drosophila*, Polo kinase is required for the generation of MPM2 epitopes (Logarinho and Sunkel, 1998). In wild-type stage 14 oocytes, many MPM2-reactive proteins are present, whereas in *cdc2*$^{E1\text{-}24}$/*cdc2*^{B47} mutants they are severely reduced, indicating that the generation of MPM2 epitopes requires Cdk1 activity in *Drosophila* oocytes. *twine*^{1} and *endos*^{00003} stage 14 oocytes also had drastically reduced MPM2 levels, suggestive of low Polo and/or low Cdk1 activity in vivo (Fig. 3F; see also Fig. S2M in the supplementary material).

Elgi, a predicted E3 ubiquitin ligase, interacts with Endos in vitro

To identify proteins that directly bind to Endos and to better understand its role in meiosis, we performed a *Drosophila* in vitro expression cloning binding screen (Fig. 4A) modified from the approach previously used to screen for kinase substrates (Lee et al., 2005). Sequence-verified cDNAs corresponding to 5856 unique *Drosophila* genes were converted into ^{35}S-labeled proteins, which were screened for binding to an Endos fusion protein. Of the two candidates that bound specifically to Endos, the predicted E3 ubiquitin ligase encoded by *CG17033* (renamed *early girl*, or *elgi*; see below) was the strongest (Fig. 4B).

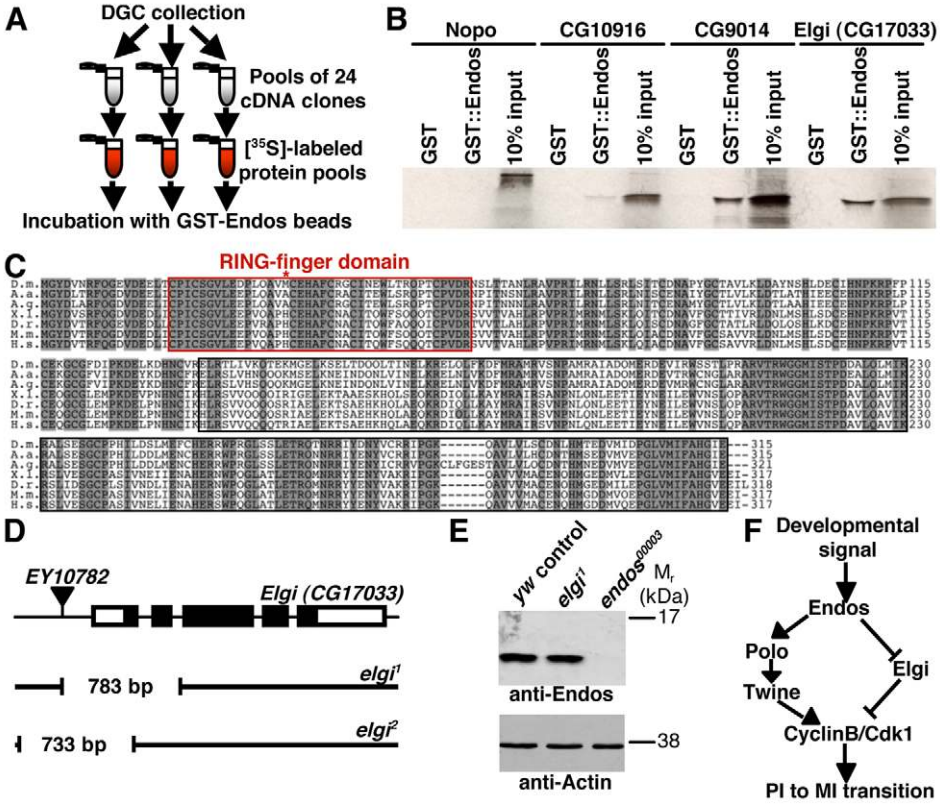

Fig. 4. Endos binds to a putative E3 ubiquitin ligase encoded by *elgi*. (**A**) DIVEC screen used to identify Endos interactors. (**B**) Autoradiogram showing that the GST::Endos fusion protein specifically binds to the closely related CG9014 and Elgi E3 ubiquitin ligases, but not to more distant E3s, such as Nopo and CG10916. (**C**) Elgi shares 81%, 80%, 57%, 57%, 56% and 54% amino acid identity with the *A. aegypti*, *A. gambiae*, *X. laevis*, *D. rerio*, *M. musculus* and *H. sapiens* homologs, respectively. Red box marks RING domain; identical amino acid residues are shaded. Asterisk indicates the first methionine for the predicted Elgi^{2} protein. (**D**) *elgi* alleles generated by imprecise excision of *EY10782*. Thick bars represent *elgi* exons (coding region in black). Gaps in black lines indicate deleted regions in *elgi*^{1} and *elgi*^{2}. (**E**) Western blot showing normal Endos levels in *elgi*^{1} ovaries. Actin was used as a loading control. (**F**) Model for the role of Endos in oocyte meiotic maturation. Endos controls Polo kinase and, perhaps indirectly, Twine levels, leading to the activation of Cyclin B/Cdk1. By binding and inhibiting Elgi, Endos may have a parallel role in refining the timing of meiotic maturation.

Elgi has a highly conserved RING finger domain and it has vertebrate homologs (Fig. 4C), including the human NRDP1 protein, which has E3 ubiquitin ligase activity in vitro (Qiu and Goldberg, 2002; Qiu et al., 2004). Remarkably, the closely related gene *CG9014* encodes a protein identified as an Endos interactor in a large-scale yeast two-hybrid screen (Giot et al., 2003). Elgi and CG9014 are the most closely related *Drosophila* E3 ligases, having 44% identity and 63% similarity at the amino acid level. We confirmed that Endos binds to Elgi and CG9014, but not to more distantly related E3 ligases (Fig. 4B), but we were unable to generate high quality anti-Elgi polyclonal antibodies to confirm the Endos-Elgi interaction in vivo. We also detected *elgi* and *CG9014* mRNA expression in heads and carcasses, but *elgi* predominates in ovaries (see Fig. S7A in the supplementary material).

elgi mutation results in premature metaphase I

To examine the role of *elgi* in meiotic maturation, we generated two deletion alleles (Fig. 4D). $elgi^{1}$ is likely a null allele because no mRNA is detected in homozygotes (see Fig. S7B in the supplementary material). The disruption of *elgi* results in semi-lethality, indicating a role during development. $elgi^{2}$ lacks a small portion of the *elgi* coding region (Fig. 4D), and some mRNA is still detected when in trans to $elgi^{1}$ (see Fig. S7B in the supplementary material). Although $elgi^{1}$ females have normal ovarian morphology and are fertile, progression from prophase I to metaphase I occurs prematurely (Fig. 1Q-V; see also Table S1 in the supplementary material), prompting the name *early girl*, or *elgi*. Nuclear envelope breakdown is also premature in $elgi^{1}$ mutants (see Fig. S3M-Q in the supplementary material), with metaphase I spindles comparable to those of control oocytes (Fig. 2F) and perhaps slightly elevated MPM2 levels (Fig. 3F; a clear increase in $elgi^{1}$ MPM2 levels was observed in two out of three experiments). $elgi^{1}/Df(3L)brm11$ hemizygotes show similar phenotypes (see Table S1 in the supplementary material), consistent with $elgi^{1}$ being a null allele. When $elgi^{1}$ is in *trans* to $elgi^{2}$, an even higher percentage of oocytes undergoes premature metaphase I, suggesting a dominant-negative effect. The premature metaphase I in *elgi* mutants in contrast to the failed metaphase I transition of $endos^{00003}$ oocytes suggests that *endos* and *elgi* play antagonistic roles in meiotic maturation.

Different models could explain how Endos and Elgi interact. E3 ubiquitin ligases in combination with E1 ubiquitin-activating and E2 ubiquitin-conjugating enzymes covalently attach ubiquitins to target proteins, thereby inducing their degradation, or modulating their subcellular localization, interaction with other proteins or activity (Pickart, 2001). It is unlikely that Endos is a direct target of Elgi because we did not observe any changes in Endos mobility or levels in $elgi^{1}$ oocytes (Fig. 4E). Endos instead may inhibit Elgi by blocking its interaction with target proteins. Because Polo kinase and Twine protein levels are reduced in $endos^{00003}$ mutants, we wished to determine whether this was due to high levels of Elgi activity. However, Twine and Polo levels are still reduced in $endos^{00003}$ $elgi^{1}$ double mutants and are unaffected in $elgi^{1}$ mutants (Fig. 3A-C; see also Fig. S6 in the supplementary material), suggesting that Elgi is not responsible for degrading these proteins. In addition, the meiotic maturation defect of $endos^{00003}$ $elgi^{1}$ double mutants is very similar to that of $endos^{00003}$ mutants (see Table S1 in the supplementary material). This is not likely to be due to redundancy between *elgi* and *CG9014* because loss of *elgi* function alone causes premature meiotic maturation (Fig. 1V). Instead, we propose that Endos controls meiotic maturation via parallel mechanisms, by modulating the protein levels of Polo kinase (and Twine) and by modulating Elgi activity (Fig. 4F).

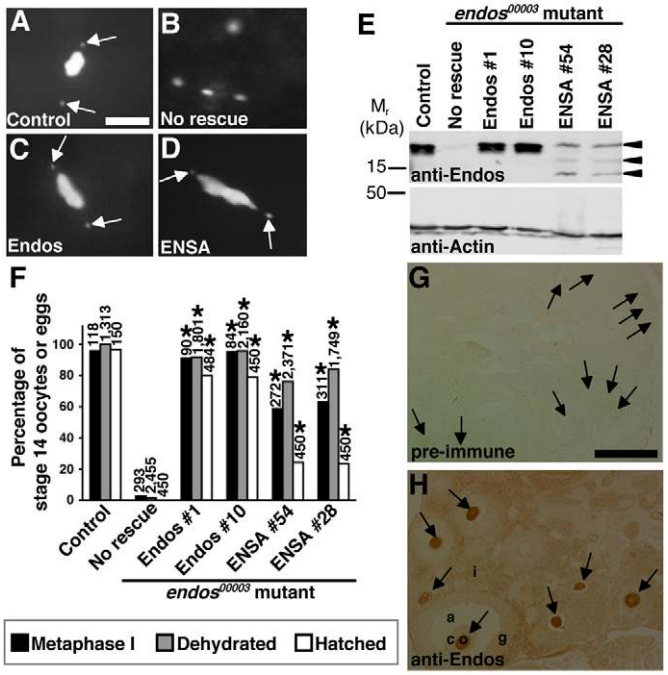

Fig. 5. The function of α-endosulfine might be evolutionarily conserved. (**A-D**) DAPI-stained stage 14 oocytes from control and $endos^{00003}$ females expressing Endos or human α-endosulfine (ENSA). (A) Control oocytes arrested in metaphase I. (B) *endos* mutant oocytes with dispersed DNA. (C,D) *nanos-Gal4::VP16*-driven germline expression of *UAS-endos* (C) or *UAS-ENSA* (D) transgenes showing *endos* rescue. Arrows indicate fourth chromosomes. Scale bar: 5 μm. (**E**) Western analysis showing ovarian expression of Endos and ENSA in rescued females. Actin was used as a loading control. Arrowheads indicate ENSA-specific bands (lower bands are probably degradation products), which are absent in the 'No rescue' control. (**F**) Quantification of $endos^{00003}$ rescue. The number of stage 14 oocytes (metaphase I arrest and dehydration) or eggs (hatch rate) analyzed is shown above the bars. **P*<0.001. (**G**,**H**) Mouse ovary immunohistochemistry showing that anti-Endos antibodies strongly label the cytoplasm of oocytes (H), whereas no signal is detected by pre-immune serum (G). o, oocyte (arrows); c, cumulus cells; g, granulosa cells; a, antrum; i, interstitial cells. Scale bar: 200 μm.

The meiotic function of *α-endosulfine* may be evolutionarily conserved

Endos is 46% identical to mammalian α-endosulfines (Drummond-Barbosa and Spradling, 2004). To determine whether *α-endosulfine* is also functionally conserved, we tested whether expression of ENSA, the human homolog, could rescue the $endos^{00003}$ meiotic maturation defect (Fig. 5A-F). When *UAS-endos* transgenes were specifically expressed in the germline of $endos^{00003}$ females, the transition to metaphase I, stage 14 dehydration, and fertility were efficiently restored (Fig. 5C,F). Remarkably, germline-driven *UAS-ENSA* transgenes also significantly rescued the $endos^{00003}$ defects (Fig. 5D,F), suggesting conservation of the molecular function of α-endosulfine.

We also investigated whether *α-endosulfine* is expressed in adult mammalian oocytes. We detected mRNA expression of *α-endosulfine* in mouse ovaries (J.R.V.S. and D.D.-B., unpublished). Our anti-Endos antibodies (generated against full-length Endos) (see Drummond-Barbosa and Spradling, 2004) recognize ENSA (see Fig. 5E), which is 93% identical to the mouse protein. We therefore

used them for immunohistochemistry, detecting strong expression of α-endosulfine protein in the cytoplasm of adult mouse oocytes (Fig. 5G,H). These data suggest that the meiotic function of α-endosulfine may have been evolutionarily conserved.

DISCUSSION

Our studies demonstrate previously unknown roles for α-endosulfine in meiotic maturation. Endos is required to ensure normal Polo kinase levels and, perhaps indirectly, to stabilize Twine/Cdc25 phosphatase. A generalized effect of *endos* on protein translation or stability is unlikely, given that Cyclin B and actin protein levels are both unaffected by the loss of *endos* function. Owing to problems in maintaining high levels of Twine or Polo transgenes in *endos* mutants, however, we could not demonstrate that the low levels of Twine and/or Polo do indeed cause the *endos* meiotic maturation defects. In addition, our data suggest that Endos has a separate role during meiotic maturation, through the negative regulation of Elgi. The function of α-endosulfine in meiotic maturation potentially may be conserved because ENSA, the human homolog, can efficiently rescue the *endos* mutant phenotype, and because α-endosulfine is expressed in mammalian oocytes. It would be interesting and informative to determine whether elimination of *α-endosulfine* function in the mouse germline results in similar meiotic maturation defects to those seen in *Drosophila* and in sterility.

Levels of Cdc25 phosphatases are tightly regulated during the cell cycle by the balance of protein synthesis and degradation (Boutros et al., 2006; Busino et al., 2004; Karlsson-Rosenthal and Millar, 2006). Phosphorylation of Ser18 and Ser116 residues by Cyclin B/Cdk1 results in mouse Cdc25A stabilization, thereby creating a positive-feedback loop that allows Cdc25A to dephosphorylate and activate Cyclin B/Cdk1. Evidence from *Xenopus* studies indicates that phosphorylation and activation of Cdc25 by Polo-like kinase generate MPM2 epitopes, which reflect high Cyclin B/Cdk1 activity (Kumagai and Dunphy, 1996; Qian et al., 2001). Moreover, a recent study in *C. elegans* demonstrates a role for Polo-like kinase in meiotic maturation (Chase et al., 2000). It is therefore likely that the low Twine levels observed in *endos* mutants are an indirect consequence of reduced Polo levels, which may result in impaired Cdk1 activity. It remains a formal possibility, however, that *endos* regulates Twine and Polo levels independently of each other. In either case, there are clear differences between the *endos* and *twine* phenotypes: only *endos* mutant oocytes show a severe reduction in Polo and a slight reduction in Cdk1 levels; *twine* but not *endos* mutants show slightly elevated Cyclin B levels; the phosphorylation status of Cdk1 seems differently altered in *endos* (appears to be hypophosphorylated) and *twine* (appears to be hyperphosphorylated, as expected) mutants relative to in control oocytes; and in vitro Cdk1 activity is reduced in immunoprecipitates from *twine* but not *endos* oocytes.

It is possible that the wild-type levels of in vitro phosphorylation of histone H1 of $endos^{00003}$ immunoprecipitates accurately reflect Cdk1 kinase activity levels in living $endos^{00003}$ oocytes, in which case we would conclude that the reduction in Twine and Polo levels observed in $endos^{00003}$ mutants is not sufficient to affect Cdk1 activity, and that the observed MPM2 epitope level reduction is simply due to low Polo levels. Another possibility is that in vitro phosphorylation of histone H1 is not reflective of the in vivo Cdk1 kinase activity levels in *endos* mutants. For example, Cdk1 substrate specificity may be altered in *endos* mutants such that endogenous substrates other than histone H1 are not properly phosphorylated, or Cdk1 kinase activity may be reduced in specific subcellular pools in these mutant oocytes, perhaps via local alterations in phosphorylation or Cyclin B levels. In fact, the spatial regulation of Cyclin B has been reported during meiosis and syncytial mitotic cycles in *Drosophila* (Huang and Raff, 1999; Swan and Schupbach, 2007).

Although we were unable to confirm the Endos-Elgi interaction in vivo, their strong interaction in vitro, combined with the premature meiotic maturation phenotype of *elgi* mutants, suggest that these genes function in the same pathway. The mammalian Elgi homolog Nrdp1 has been shown to act as an E3 ubiquitin ligase in vitro to promote degradation of the Erbb3 and Erbb4 receptor tyrosine kinases (Qiu and Goldberg, 2002), and of the inhibitor-of-apoptosis protein BRUCE (Qiu et al., 2004). It would be interesting to determine whether Elgi also has E3 ligase activity in flies and to identify its direct targets. *Nrdp1* mRNA is expressed in multiple human tissues, including the ovary (Qiu and Goldberg, 2002); however, a role for NRDP1 in meiotic maturation or the modulation of Cdk1 has not been examined. The strong degree of amino acid similarity between human NRDP1 and Elgi is suggestive of functional conservation.

The premature entry into metaphase I observed in *elgi* null mutants in the absence of effects on Polo or Twine levels suggests that Endos uses a separate mechanism that involves Elgi function to control the timing of Cdk1 activation and, ultimately, that of meiotic maturation, without necessarily affecting the final levels of Cdk1 activation. The premature meiotic maturation phenotype of *elgi* mutants is reminiscent of the phenotype recently reported for *matrimony* heterozygous mutants (Xiang et al., 2007). In these studies, Matrimony was reported to interact with Polo kinase in vivo and to function as a Polo inhibitor, with a suggested role in finely controlling the timing of meiotic maturation. One possible model to explain the premature meiotic maturation of *elgi* mutant oocytes is that Elgi positively regulates the interaction between Matrimony and Polo, and that Endos controls the precise timing of meiotic maturation by inhibiting this E3 ubiquitin ligase, in addition to having a key role in promoting high Polo (and Twine) protein levels. It will be very interesting to experimentally address this possibility in future studies.

In addition to having key roles in meiosis, we also found that *Drosophila α-endosulfine* is required during early embryonic mitoses. These findings are consistent with recent studies showing, as part of a large-scale screen for genes required for mitotic spindle assembly in *Drosophila* S2 cells, that disruption of *α-endosulfine* expression by RNA interference produces defects such as chromosome misalignment and abnormal spindles (Goshima et al., 2007). It is conceivable that α-endosulfine uses similar mechanisms in both meiosis and mitosis. Further characterization of the role of *α-endosulfine* in mitosis will help to address this question.

Given the central role that we report for Endos in meiotic maturation and the fact that Endos is expressed throughout oogenesis, it will next be essential to investigate how Endos activity is regulated as the oocyte develops and becomes competent to undergo meiotic maturation. Intriguingly, Endos contains a highly conserved protein kinase A (PKA) phosphorylation site. Indeed, mammalian homologs can be phosphorylated by PKA at this site (Dulubova et al., 2001), and, in vertebrate oocytes, high levels of cyclic adenosine monophosphate (cAMP) and PKA activity inhibit the resumption of meiosis by inhibiting Cyclin B/Cdk1 activity (Burton and McKnight, 2007; Kovo et al., 2006). Upon oocyte meiotic maturation, cAMP levels and PKA activity decrease (Burton and McKnight, 2007; Kovo et al., 2006). Although the evidence suggests that PKA-dependent phosphorylation is responsible for

activation of the Cdk1-inhibitory kinase Wee1 and for inactivation of the Cdk1-activating phosphatase Cdc25 (Burton and McKnight, 2007), it is possible that PKA has additional roles in controlling meiotic maturation, perhaps via α-endosulfine. In fact, two forms of Endos with different electrophoretic mobilities are present in *Drosophila* ovaries (Drummond-Barbosa and Spradling, 2004), with the lower mobility form being specifically present in stage 14 oocytes (J.R.V.S. and D.D.-B., unpublished). However, it remains to be determined whether these different forms of Endos are caused by phosphorylation, and, if so, what the effect of phosphorylation is on Endos activity.

Finally, although this was not the focus of these studies, some of our results suggest that Endos does not regulate insulin secretion (see Fig. S1 in the supplementary material), which is different from mammalian studies that link α-endosulfine to this process (Virsolvy-Vergine et al., 1988; Virsolvy-Vergine et al., 1992). It is possible that this discrepancy results from differences in the function of α-endosulfine between species, perhaps reflecting an evolutionarily newer role of α-endosulfine in the control of insulin secretion. It is important, however, to emphasize that the role of α-endosulfine in insulin secretion has not been tested in vivo. Nevertheless, human *α-endosulfine* mRNA is expressed in multiples tissues, including heart, brain, lung, pancreas, kidney, liver, spleen, and skeletal muscle (Heron et al., 1998), and we show herein that it is also expressed in the ovary. The wide range of expression of human *α-endosulfine* suggests that it is likely to play multiple biological roles, perhaps including, as our studies point to, a potential role in meiotic maturation.

J.R.V.S. and D.D.-B. designed and interpreted the experiments, and wrote the manuscript. J.R.V.S. performed all *Drosophila* experiments, including the DIVEC screen. J.R.V.S. and B.C. performed live imaging. S.T. conducted the mouse ovary immunohistochemistry and provided mouse cDNAs, with support from S.K.D. L.A.L. conceived of and established the DIVEC methodology for binding screens, and provided radiolabeled protein pools for the primary screen. We are grateful to E. Lee for expert advice on the DIVEC screen, and for the *pCS2* vector, and to T. Murphy for *pUASpI*. We thank M. Fuller, R. S. Hawley, and the Bloomington Stock Center for *Drosophila* stocks, and C. Sunkel, J. Großhans and the Developmental Studies Hybridoma Bank for antibodies. We thank L. Zhang, K. LaFever and T. Daikoku for technical assistance, and L. LaFever for making the *UAS-endos* lines. Thanks to D. Miller and members of his lab for help with Nomarski microscopy, and to C. Spencer and S. Von Stetina for help with movie assembly. We are grateful to H.-J. Hsu, E. T. Ables, L. LaFever, and anonymous reviewers for valuable comments on the manuscript. This work was supported by National Institutes of Health grants GM069875 (D.D.-B.), GM074044 (L.A.L.) and HD12304 (S.D.), and training grants 2T32HD007502 (support for J.R.V.S.) and 2T32HD007043 (support for J.R.V.S. and S.T.).

Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/135/22/3697/DC1

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