THE ROLE OF α-ENDOSULFINE IN THE FEMALE MEIOTIC CELL CYCLE IN *DROSOPHILA*

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ABC- ATP-binding cassette

AKT/PKB- Protein Kinase B

APC/C- anaphase promoting complex/cyclosome

aret- arrest

ATP- adenosine triphosphate

Bru- Bruno

CAK- Cdk-activating kinase

CamKII- Ca^{2+}/calmodulin-dependent protein kinase II

cAMP- cyclic adenosine monophosphate

Cdc25- cell division cycle 25

Cdk- Cyclin-dependent kinase

C. elegans- Caenorhabditis elegans

Chk- checkpoint protein kinase

COX- cyclooxygenase

CRM1- Chromosome Region Maintenance 1

CRS- Cytoplasmic Retention Sequence

CSF- Cytostatic Factor

C-TAK- Cdc25 associated protein kinase

DAPI- 4',6-diamidino-2-phenyllindole

D-box- Destruction-box

DGC- Drosophila Gene Collection

DIVEC- Drosophila in vitro expression cloning

Elgi- Early girl

Emi1- Early mitotic inhibitor 1

Endos- Endosulfine

ERK- extracellular signal-regulated kinase-1

ESC- escort stem cell

FITC- fluorescein isothiocyanate

Gwl- Greatwall

GPCR- G-protein-coupled receptor

GSC- germline stem cell

K^{ATP}- ATP-dependent potassium

Kir- potassium inward rectifying

KEN- Lysine (K), Glutamic acid (E), Asparagine (N)

LH- Luteinizing hormone

MAPK- mitogen-activated protein kinase

MAT1- Ménage a Trois

MPF- maturation/M-phase promoting factor

MPM2- Mitotic Protein Monoclonal 2

MSP- Major Sperm Protein

MTOC- microtubule organizing center

Mtrm- Matrimony

M-phase- Mitosis/Meiosis phase

MudPIT- multidimensional protein identification technology

NES- nuclear export sequence

NLS- nuclear localization sequence

Nrdp1- neuregulin receptor degrading protein 1

1-MeAde- 1-methyladenine

PDE- phosphodiesterase

PGC- primordial germ cell

PHHI- Persistent Hyperinsulinemic Hypoglycemia of Infancy

PKA- protein kinase A

Plk- Polo-like kinase

PP1- Protein Phosphatase 1

PP2A- type-2A Protein Phosphatase

PBD- Polo-box domain

REC8- recombination 8

RING- really interesting novel gene

RNAi- RNA-mediated interference

Scant- Scott of the Antartic

SCF- Skp1/cullin/F-box

Ser- serine

Sgo- Shugoshin

SSC- somatic stem cell

S2- Schneider 2

SMC- Structural Maintenance Chromosome

Thr- threonine

Tyr- tyrosine

Twe- Twine

SUR1- Sulfonylurea Receptor 1

CHAPTER I

BACKGROUND AND SIGNIFICANCE

The production of haploid gametes (eggs and sperm) is indispensable for eukaryotic sexual reproduction. This process is achieved by a specialized type of cell division known as meiosis. The meiotic cell divisions are tightly coordinated with the development of the gametes and must be well regulated to avoid aneuploidy (wrong chromosome number) or other chromosomal abnormalities. Down Syndrome, for example, which is caused by trisomy of chromosome 21, occurs in approximately 1 in every 1,000 live births (Egan et al., 2004; Morris et al., 2002). Thus, understanding the control and regulation of meiosis is relevant to key human health issues.

This Background and Significance Chapter will review the major developmental and regulatory events, signaling pathways and molecular players involved in oocyte meiotic maturation and arrest in different species. The last section will focus on what is currently known about *Drosophila* meiosis and will describe the previously known *in vivo* and *in vitro* functions of *α-endosulfine* in mammals and flies.

Meiosis is a specialized cell division

Following pre-meiotic DNA replication (S phase), germ cells undergo two successive nuclear divisions (Meiosis I and Meiosis II) with no intervening DNA synthesis (S phase) that ultimately lead to the reduction in chromosome number and ploidy. The first meiotic division (Meiosis I) is divided into five phases. During the first

phase (prophase I), which is further subdivided into five sequential stages (leptotene, zygotene, pachytene, diplotene, diakinesis), key meiotic events take place, including chromosome condensation, pairing and synapsis of homologous chromosomes, and exchange of genetic material between the homologs, which creates chiasmata (Alberts, 2002; Gilbert, 2006). Following these events, the homologous chromosomes align at the metaphase plate held together by the chiasmata combined with cohesion between sister chromatid arms and centromeres in metaphase I (Alberts, 2002; Gilbert, 2006). In anaphase I, sister chromatid arm cohesion is lost and homologous chromosomes separate from each other and move toward opposite poles, while sister chromatids are still linked by cohesion at the centromeres (Alberts, 2002; Gilbert, 2006). At telophase I, the homologous chromosomes complete their migration and acquire a nuclear envelope (Alberts, 2002; Gilbert, 2006). In animal cells, two new daughter cells containing a single set of replicated chromosomes are formed through cytokinesis (Alberts, 2002; Gilbert, 2006).

Meiosis II is also divided into similar phases: prophase II, metaphase II, anaphase II, telophase II, and cytokinesis. During this second cell division, however, cohesion at the centromeres is lost and the sister chromatids segregate from one another to produce four haploid daughter cells (Alberts, 2002; Gilbert, 2006). Thus, meiosis I and meiosis II, respectively, are fundamental to promote genetic diversity and to create the haploid state in the resulting daughter cells that is required to restore the parental ploidy upon fertilization.

Errors during meiosis affect the resulting gametes

Meiosis is a highly organized and regulated process. When this regulation is disturbed, chromosomal abnormalities occur that give rise to zygotes with an incorrect number of chromosomes. Thus, meiotic errors are the major cause of genetic diseases and infertility in humans (Hassold et al., 2007; Hunt and Hassold, 2008; Jones, 2008). During non-disjunction, for example, the homologous chromosomes or the sister chromatids fail to separate properly during the meiotic cell division I or II, respectively. As a result from these segregation errors, the daughter cell(s) lack a chromosome (monosomy) or gain a chromosome (trisomy), a condition defined as aneuploidy (Eric C. R. Reeve, 2001). Generally, aneuploidy leads to embryonic lethality, but in some cases the embryo survives. The most common example is Down's syndrome, which is caused by an extra copy of chromosome 21 from one of the parents (Hernandez and Fisher, 1996; Korenberg, 1993; Korenberg et al., 1994; Lejeune, 1964).

Many of the errors in meiotic chromosome segregation originate during oogenesis. In fact, maternal age is the only well-established factor associated with high incidence of meiotic non-disjunction and aneuploidy (Eichenlaub-Ritter, 1998; Eichenlaub-Ritter, 2002; Hassold and Hunt, 2001; Jones, 2008). Interestingly, age-dependent meiotic non-disjunction has also been demonstrated in genetic systems such as *Drosophila* (Jeffreys et al., 2003). The high susceptibility to meiotic errors of the oocyte (20% aneuploidy in oocytes versus 1-2% in sperm) (Hassold and Hunt, 2001; Martin et al., 1991; Pacchierotti et al., 2007) highlights the importance of understanding female gamete development.

Meiosis and gametogenesis are coordinated during development

Meiosis is tightly coupled to gamete development. After fertilization, a small group of cells in the developing mammalian embryo are induced to become primordial germ cells (PGCs), which migrate to the embryonic gonad (known as the genital ridge in mouse) (Molyneaux and Wylie, 2004; Monk and McLaren, 1981). Upon arrival, these cells differentiate into oogonia or spermatogonia depending on whether the gonad has begun to develop into an ovary or a testis, respectively (reviewed in (Blecher and Erickson, 2007). In males, spermatogenesis and meiosis do not begin until puberty and proceed without any arrest. At puberty, spermatogonia proliferate by mitosis into primary spermatocytes, which then enter meiosis. After division I of meiosis, primary spermatocytes produce two secondary spermatocytes, which then complete meiosis II to form four haploid spermatids. The process of spermatogenesis is completed by the differentiation of spermatids into mature sperm. In contrast to females, which are born with a finite number of oocytes (see below), in males, spermatogonial stem cells support sperm production throughout life (Alberts, 2002; Gilbert, 2006). (Note: because my focus in this thesis work is on female meiosis, I will not further discuss male meiosis)

During mammalian oogenesis, oogonia undergo several rounds of mitotic divisions to produce many (millions in the human embryo) oogonial germ cells before birth (Alberts, 2002; Gilbert, 2006). After these divisions, the oogonial cells become primary oocytes, undergo pre-meiotic S phase, and enter meiosis I (Figure 1.1 A). Primary oocytes progress through prophase I and subsequently arrest at the diplotene stage until just prior to ovulation (Borum, 1961) (Figure 1.1 A). Thus, females are born with a fixed reserve of primary oocytes (Pepling, 2006; Zuckerman, 1951) arrested at

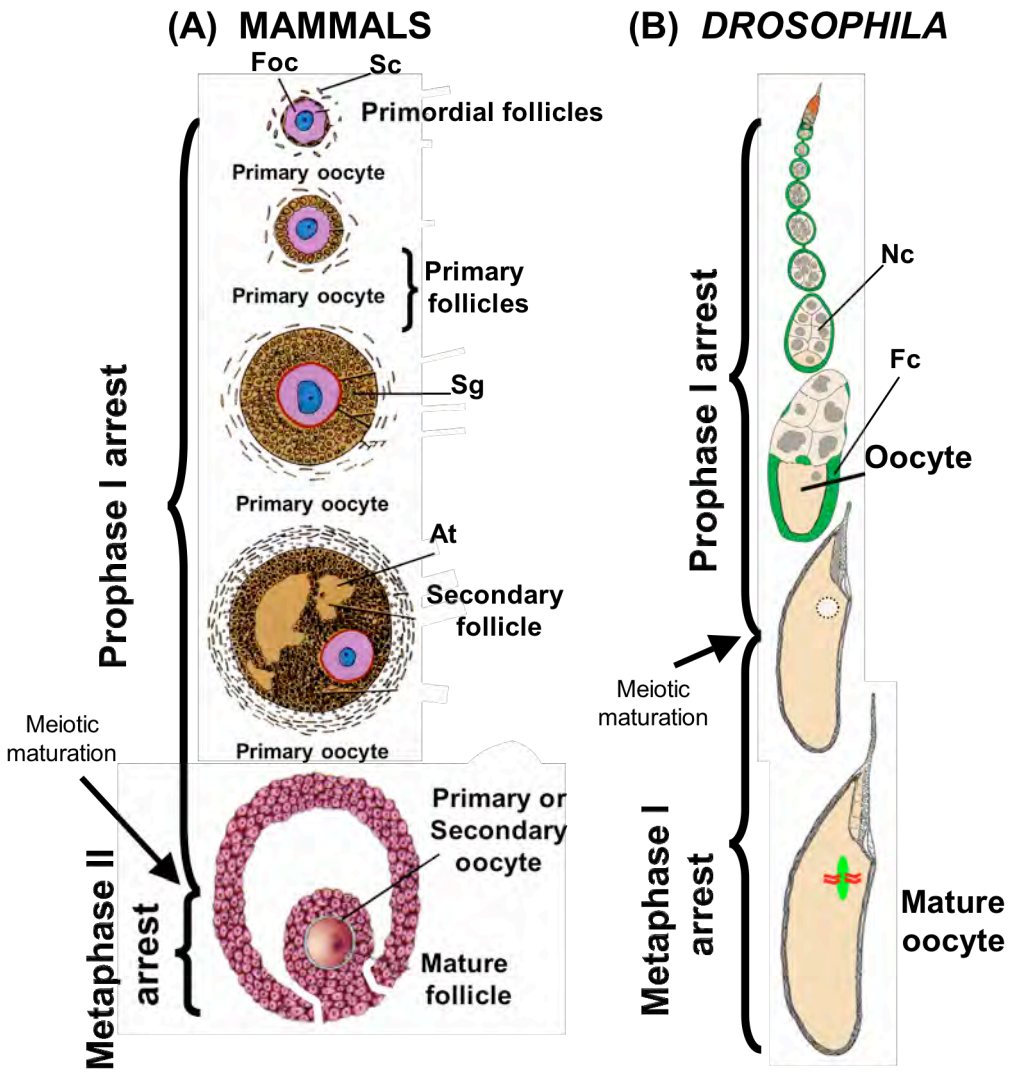

Figure 1.1. Comparison between mammalian and *Drosophila* oogenesis. (A) In humans, primordial germ cells give rise to oogonia, which become primary oocytes that enter meiosis. Interestingly, a study in mouse showed that the early female germline develops in cysts similar to what occurs in fly ovaries (Pepling and Spradling,1998). Primary oocytes arrest at prophase I before birth and remain arrested until puberty (aproximately 15 years). Upon luteinizing hormone stimulation, primary oocytes undergo meiotic maturation and produce secondary oocytes that undergo a second arrest at metaphase II. After ovulation, fertilization triggers resumption of meiosis. (B) In *Drosophila,* the oocyte within a 16-cell germline cyst enters meiotic prophase I and remains arrested for most of oogenesis (approximately 2 days). At stage 13, upon a developmental signal (unknown; potentially prostaglandins) the oocyte undergoes maturation and enters metaphase I. Upon ovulation, rehydration and mechanical pressure as the oocyte travels in the oviduct trigger completion of meiosis in *Drosophila*. Sc, Stromal cell; Foc, Follicular cell; Sg, Stratum granulosa; At, Antrum; Fc, Follicle cell; Nc, Nurse cell.

prophase I. Upon appropriate hormonal stimulation, primary oocytes undergo meiotic maturation, complete the first meiotic division and produce a first polar body and a secondary oocyte (Alberts, 2002; Gilbert, 2006). Whereas the first polar body eventually degenerates, the secondary oocyte enters meiosis II and arrests at metaphase II. Upon fertilization, the secondary oocyte completes the second division and generates a second polar body (which also degenerates) and a haploid mature egg (the ovum) (Alberts, 2002; Gilbert, 2006).

Cdk1/Cyclin B is a conserved factor essential for cell cycle progression and arrest

Discovery of Cdk1/Cyclin B

In both vertebrate and invertebrate systems, there is strong experimental evidence demonstrating that the levels of activity of maturation/M phase-promoting factor (MPF) are critical in regulating key meiotic events such as oocyte meiotic maturation and arrest. Thirty-seven years ago, Masui and Markert discovered, in the frog *Rana pipiens*, an activity in the cytoplasm of progesterone-treated oocytes that induced meiotic maturation (see below) when microinjected into immature G2 or prophase I-arrested oocytes. They coined this activity maturation/M phase-promoting factor. Seventeen years later, MPF was characterized as a heterodimer composed of a catalytic subunit, the serine/threonine cyclin dependent kinase Cdk1 (also known as Cdc2), and a regulatory subunit, Cyclin B (Dunphy et al., 1988; Gautier et al., 1990; Gautier et al., 1988; Lohka et al., 1988). To date, Cdk1 (and associated cyclins) is considered a highly conserved factor that plays essential regulatory roles in meiosis and mitosis.

Cdk1/Cyclin B activity is regulated at multiple levels during the cell cycle

Regulation of the activity of Cdk1/Cyclin B occurs at many levels involving both its catalytic and its regulatory subunits. Cdk1/Cyclin B activity also depends on the spatial and temporal regulation of its activators and inhibitors. In the following subsections, I will describe the many events that encompass this complex regulation during the cell cycle.

Cdk1 activation is first triggered by association with cyclins and activating phosphorylation

Cdk initial activation is mediated by two main events. The first event involves the association of Cdk with the cyclin regulatory subunit. Cyclin binding induces important conformational changes in the structure of the kinase. Binding of the cyclin to the PSTAIR helix (structure that contains the conserved amino acids P S T A I R) at the amino terminus of Cdk promotes helix realignment toward the catalytic cleft thereby bringing residues involved in adenosine triphosphate (ATP) binding together (Pavletich, 1999). Normally, in monomeric Cdk molecules the T-loop (structural loop in the activating domain of kinases) hinders substrate binding by blocking the catalytic site (known as the “closed” conformation) (De Bondt et al., 1993). Upon cyclin binding, the T-loop is displaced to allow access of substrates to the active site (known as the “open” conformation) and expose the activating phosphorylation site on the T-loop (Pavletich, 1999). Beyond this activating role, cyclins can also promote activity of Cdk1/Cyclin B toward specific substrates or subcellular localizations (see below) (Morgan, 1995).

The second event required to complete Cdk initial activation is the phosphorylation of a conserved threonine residue (Thr161) within the T-loop of Cdk by

Cdk-activating kinase (CAK). CAK is also a cyclin dependent kinase that consists of a catalytic subunit, Cdk7 (originally named $p40^{MO15}$), and a regulatory subunit, cyclin H (Fesquet et al., 1993; Fisher and Morgan, 1994; Makela et al., 1994; Poon et al., 1994). A third subunit, "Ménage a Trois" (MAT1), is an assembly factor that associates with Cdk7 and cyclin H (Devault et al., 1995; Fisher et al., 1995; Tassan et al., 1995). Interestingly, unlike other Cdks, CAK activity remains constant throughout the cell cycle in different systems (Brown et al., 1994; Matsuoka et al., 1994; Poon et al., 1994; Tassan et al., 1994). Some evidence, however, points to regulation in the levels of Thr161 phosphorylation during embryonic cycles (Edgar et al., 1994). Several studies have proposed that the Thr161 phosphorylation is essential for the catalytic activity of Cdk and/or for substrate binding (Kaldis, 1999; Russo et al., 1996).

Cdk1/Cyclin B activation is regulated by the antagonistic functions of Wee1/Myt1 kinases and the dual specificity phosphatase Cdc25

Cdk1/Cyclin B is maintained in an inactive state by the phosphorylation of Thr14 and Tyr15 residues within the ATP-binding site of the catalytic Cdk1 subunit. Whereas Thr14 phosphorylation interferes with ATP binding, phosphorylation at Tyr15 interferes with the transfer of phosphate to the bound substrate (Stein, 2004). Wee1 and Myt1 are two related conserved kinases that catalyze the phosphorylation of these negative regulatory sites to inhibit M-phase entry. Wee1 phosphorylates the tyrosine residue homologous to Tyr15 in many systems from yeast to mammals (Mueller et al., 1995a; Parker et al., 1991; Parker and Piwnica-Worms, 1992; Piwnica-Worms et al., 1991; Stumpff et al., 2004). During interphase, Wee1 is found predominantly in the nucleus, where it excludes the cytoplasmic activated Cdk1/Cyclin B, but it translocates to the

cytoplasm at the G2/M transition (Baldin and Ducommun, 1995; Heald et al., 1993). In contrast, Myt1 is a Golgi membrane-associated kinase that remains in the cytoplasm and phosphorylates both Thr14 and Tyr15 Cdk1 sites (Booher et al., 1997; Fattaey and Booher, 1997; Liu et al., 1997; Mueller et al., 1995b; Wells et al., 1999). In addition to inactivating Cdk by its dual phosphorylation, Myt1 binds and sequesters active Cdk1/Cyclin B in the cytoplasm (Liu et al., 1999; Wells et al., 1999). Similar to Cdc25 (see below), Wee1/Myt1 activities are regulated by phosphorylation and by 14-3-3 binding (reviewed in (Perry and Kornbluth, 2007).

Cell cycle progression is triggered when the inhibitory functions of Wee1/Myt1 kinases on Cdk1/Cyclin B are counteracted by the dual enzymatic activity of the conserved Cdc25 phosphatase. Multiple Cdc25 isoform are present in different species including mammals (Cdc25A, Cdc25B, Cdc25C), *Xenopus* (XCdc25A, XCdc25C), *C. elegans* (Cdc25.1, Cdc25.2, Cdc25.3, Cdc25.4) and *Drosophila* (Twine, String). In all of these systems, Cdc25 is the phosphatase that removes both Tyr15 and Thr14 inhibitory phosphorylations from Cdk1 (Dunphy and Kumagai, 1991; Gautier et al., 1991; Gould and Nurse, 1989; Lee et al., 1992; Millar et al., 1991; Sigrist et al., 1995; Strausfeld et al., 1991), and thus is essential to activate the Cdk1/Cyclin B complex (Boutros et al., 2006; Takizawa and Morgan, 2000). Due to its essential functions during the cell cycle, Cdc25 activity is highly regulated post-translationally by phosphorylation, protein binding and degradation.

Phosphorylation status regulates Cdc25 activity

Activation

The activation of Cdc25 is regulated by multiple phosphorylation and dephosphorylation events. The Cdc25 amino terminus is phosphorylated at the Thr130 (humans) or Thr138 (*Xenopus*) residue (Izumi and Maller, 1993). Phosphorylation at Thr138/130 releases the 14-3-3 proteins (a family of conserved regulatory molecules) (bound to Ser287) from Cdc25 during the G2/M transition (Margolis et al., 2003). It has been suggested that Thr138 phosphorylation might decrease the affinity of Cdc25 for 14-3-3 and thus might expose the Ser287 to be readily dephosphorylated (Perry and Kornbluth, 2007). Although phosphorylation of human Thr130 can be mediated by Cdk1/Cyclin B (Strausfeld et al., 1994), *Xenopus* Thr138 is phosphorylated by the cyclin dependent kinase 2 (Cdk2) (Izumi and Maller, 1993; Margolis et al., 2003) and by extracellular signal-regulated kinase-1 or Mitogen-activated protein kinase (ERK-MAPK) during oocyte maturation (Wang et al., 2007). It is not clear how Thr138 (130) phosphorylation leads to the release of 14-3-3.

Following the removal of 14-3-3 from Cdc25, dephosphorylation of the inhibitory Ser287 residue further contributes to Cdc25 activation. Recent studies have demonstrated that Protein Phosphatase 1 (PP1) is the phosphatase responsible for Cdc25C dephosphorylation at Ser287 (Margolis et al., 2003). These studies also indicated that dephosphorylation at this site can only occur after the release of 14-3-3 (consistent with the above hypotheses). The exact order and timing of these events, however, is not fully understood.

The initial activation of Cdc25 is based on a linear phosphorylation pathway. A second feedback amplification pathway, however, is key in promoting the full activation of Cdc25. Once Cdk1/Cyclin B is dephosphorylated and activated by Cdc25, Cdk1/Cyclin B amplifies its activity by participating in a positive feedback loop and mediating the phosphorylation, further activation and stability of Cdc25 (Busino et al., 2004; Hoffmann et al., 1993; Karlsson-Rosenthal and Millar, 2006; Kumagai and Dunphy, 1992). Phosphorylation of residue Ser285 (*Xenopus*) (Ser214 in humans) on Cdc25 by Cdk1/Cyclin B, for example, plays an important regulatory role by preventing the Chk1-mediated rephosphorylation of Ser287 and by enhancing the interaction between PP1 and Cdc25 (Bulavin et al., 2003a; Bulavin et al., 2003b; Margolis et al., 2006b). Cdk1/Cyclin B also controls phosphorylation of other Cdc25 sites (Thr48, Thr67, Thr205), which, based on mutational analysis, are essential for Cdc25 activation (Perry and Kornbluth, 2007).

The conserved Polo-like kinase (Plk) is another key Cdc25 activator that participates in the Cdk1/Cyclin B-Cdc25 amplification loop (Abrieu et al., 1998; Karaiskou et al., 1999; Roshak et al., 2000). In the absence of Cdk1 and Cdk2 kinase activities, *Xenopus* extracts were found to have another kinase that phosphorylated and activated XCdc25C (Izumi and Maller, 1995). Subsequently, *Xenopus* Polo-like kinase (Plx), was found to phosphorylate Cdc25 and increase its activity *in vitro* (Kumagai and Dunphy, 1996). The relevance of this phosphorylation was demonstrated when expression of a constitutively active Plx (T201D) in *Xenopus* oocytes was sufficient for *in vitro* activation of XCdc25C and entry into M phase (Abrieu et al., 1998; Kumagai and Dunphy, 1996; Qian et al., 1998). In addition, the activation of XCdc25C and Cdk/Cyclin

B was suppressed by the immunodepletion of Plx from *Xenopus* extracts (Abrieu et al., 1998).

Inactivation

During interphase, Cdc25 is kept inactivated in the cytoplasm due to the phosphorylation of its Ser287 (*Xenopus*) (Ser216 in humans) residue. It is also well established that in response to DNA damage, Cdc25 is phosphorylated at Ser287 by the checkpoint kinases Chk1 and Chk2 (Furnari et al., 1999; Graves et al., 2001; Kumagai and Dunphy, 1999; Kumagai et al., 1998; Lopez-Girona et al., 1999; Peng et al., 1997; Yang et al., 1999). Phosphorylation at this serine inhibits Cdc25 function by creating an interaction site for 14-3-3 proteins and thus inducing its sequestration in the cytoplasm. As a consequence, the cell arrests at the G2 phase until DNA repair is completed. It has been demonstrated that Ser287 phosphorylation and binding of 14-3-3 proteins are also required to maintain the normal G2 arrest in oocytes (Kumagai et al., 1998; Nakajo et al., 1999; Oe et al., 2001; Peng et al., 1997). These studies have led to the proposal that binding of 14-3-3 proteins inhibits Cdc25 function in the cytoplasm, perhaps by interfering with the interaction of Cdc25 with activators (e.g. Polo kinase; see below) and/or substrates (e.g. Cdk1/Cyclin B) (discussed in (Nebreda and Ferby, 2000; Perdiguero and Nebreda, 2004). In addition to the normal Chk1-dependent phosphorylation (Nakajo et al., 1999; Oe et al., 2001; Peng et al., 1997), Cdc25-associated protein Kinase (C-TAK) (Peng et al., 1998), protein kinase A (PKA) (Duckworth et al., 2002; Schmitt and Nebreda, 2002), and the Ca^{2+}/calmodulin-dependent protein kinase II (CamKII) (Hutchins et al., 2003) have also been shown to phosphorylate the Ser287/216 residue of Cdc25.

Dephosphorylation by the type-2A Protein Phosphatase (PP2A) has also been implicated in the inactivation of Cdc25 (Clarke et al., 1993; Karaiskou et al., 1999; Margolis et al., 2006a). Studies with *Xenopus* egg extracts have found that PP2A removes the activating Cdk1/Cyclin B phosphorylation from XCdc25C and thus maintains XCdc25C in a dephosphorylated, low activity state (Clarke et al., 1993). In addition, experiments using oocyte extracts have demonstrated that PP2A antagonizes the Polo-mediated activation of Cdc25 (Karaiskou et al., 1999). Based on the analysis of the function of PP2A during the DNA damage checkpoint, it has also been predicted that PP2A dephosphorylates the conserved threonine (Thr138) (see next) on Cdc25 and indirectly inhibits the release of the inhibitory 14-3-3 proteins during the normal cell cycle (Margolis et al., 2006a).

Cdc25 subcellular localization is modulated during the cell cycle and is required for Cdk1/Cyclin B nuclear activation

As discussed above, binding of 14-3-3 proteins sequesters Cdc25C in the cytoplasm and interferes with its activation. In addition to the regulation by 14-3-3 binding, Cdc25 localization is also controlled by its nuclear localization (NLS) and nuclear export (NES) sequences (Kumagai and Dunphy, 1999; Yang et al., 1999). Evidence suggests that Cdc25 shuttles between the nucleus and the cytoplasm during interphase (Kallstrom et al., 2005; Yang et al., 1999). At the G2/M transition, however, the nuclear localization predominates due to the inhibition of 14-3-3 binding by dephosphorylation of the Ser287 residue and inhibition of Cdc25 export (Bahassi el et al., 2004; Kumagai and Dunphy, 1999; Toyoshima-Morimoto et al., 2002). Based on the outcome from experiments using a S287A XCdc25C mutant that cannot bind 14-3-3

proteins, it was proposed that 14-3-3 binding markedly reduces XCdc25C nuclear translocation due to a decreased ability of XCdc25C to bind to its nuclear import receptor importin-α (Kumagai and Dunphy, 1999; Yang et al., 1999). One hypothesis that has been proposed from these findings is that 14-3-3 binding to Ser287/216 inhibits nuclear import by blocking importin-α/β access to the Cdc25 NLS, which happens to be very close (amino acids 298-316), and enhances the rate of Chromosome Region Maintenance 1 (CRM1)-mediated nuclear export by the NES.

Recent studies have also determined that activating phosphorylation by Polo-like kinases blocks residues in the NES of Cdc25 and thus promote its nuclear translocation (Bahassi el et al., 2004; Toyoshima-Morimoto et al., 2002). The current model predicts that nuclear localization of Cdc25 is required to counteract the function of Wee1, activate nuclear Cdk1/Cyclin B, and thus initiate the events inside the nucleus that lead to nuclear envelope breakdown and M-phase progression (Takizawa and Morgan, 2000).

Phosphorylation of Cyclin B controls the activity of Cdk1/Cyclin B by promoting its nuclear translocation

The kinase functions of Cdk1/Cyclin B are also triggered by the control of its subcellular localization by Cyclin B phosphorylation. Similar to Cdc25, Cyclin B localization is determined by the relative rates of nuclear export and import, which are differentially established during interphase or upon M-phase entry (Hagting et al., 1998; Toyoshima et al., 1998; Yang et al., 1998). The cytoplasmic localization of Cyclin B at interphase depends on a Cytoplasmic Retention Sequence (CRS) on its amino terminus (Pines and Hunter, 1994). The CRS also contains a NES that can bind the export factor (CRM1) thereby contributing to the Cdk1/Cyclin B high export rate during interphase

(Hagting et al., 1998; Toyoshima et al., 1998; Yang et al., 1998). However, during the G2/M transition, phosphorylation of serine residues in the CRS/NES of Cyclin B blocks CRM1 binding and inhibit the nuclear export of Cdk1/Cyclin B and/or enhance its nuclear import rates (Hagting et al., 1999; Hagting et al., 1998; Li et al., 1995; Li et al., 1997; Toyoshima et al., 1998; Yang et al., 2001).

Interestingly, Cyclin B does not contain a canonical NLS but seems to employ two nuclear import mechanisms. The first mechanism is unusual because it involves the transporter importin-β and thus does not require importin-α, and also is Ran independent (Moore et al., 1999; Takizawa et al., 1999). Although this mechanism is not completely understood it seems to be unrelated to the CRS (Moore et al., 1999). The second mechanism requires activating phosphorylation in the CRS to drive Cdk1/Cyclin B high import rates (Hagting et al., 1999; Li et al., 1997).

Some of the kinases involved in phosphorylating and regulating Cyclin B localization have been identified. Plk-1 phosphorylates serine residues (Ser147 in *Xenopus*; Ser198 in humans) in the NES of Cyclin B and inactivates its nuclear export (Toyoshima-Morimoto et al., 2002; Toyoshima-Morimoto et al., 2001). Mammalian Plk-1 was also shown to phosphorylate serine residues in the CRS of Cyclin B, in cooperation with MAPK (Erk2) and Cdk1/Cyclin B kinases, and trigger rapid import of Cyclin B (Yuan et al., 2002). Thus, phosphorylation in the CRS/NES of Cyclin B promotes the translocation of Cdk1/Cyclin B into the nucleus, a process that is required for nuclear envelope breakdown.

Polo-like kinases regulate the cell cycle at many levels

The *polo* gene was originally identified in *Drosophila* and was shown to encode a conserved serine/threonine kinase required for mitosis and meiosis (Llamazares et al., 1991; Sunkel and Glover, 1988). Homologs of the polo-like kinase (Plk) have since been identified in many other species including *Saccharomyces cerevisiae* (Cdc5p) (Kitada et al., 1993), *Schizosaccharomyces pombe* (plo1+) (Ohkura et al., 1995), *Xenopus laevis* (Plx1, Plx2, Plx3) (Duncan et al., 2001; Kumagai and Dunphy, 1996), *Caenorhabditis elegans* (Plk-1) (Chase et al., 2000; Ouyang et al., 1999), mouse (Plk) (Donohue et al., 1995; Lake and Jelinek, 1993; Simmons et al., 1992) and humans (Plk1) (Golsteyn et al., 1994; Hamanaka et al., 1995; Li et al., 1996). Studies in *Drosophila* embryos determined that during interphase Polo is localized in the cytoplasm but then redistributes during mitosis to the condensed chromosomes (Llamazares et al., 1991).

In addition to the conserved amino-terminal kinase domain, Plks have a unique conserved Polo-box domain at the carboxy terminus (Lowery et al., 2004; Nigg, 1998). The Polo-Box domain (PBD) plays important *in vivo* roles in regulating Plk localization and function by acting as a phosphopeptide-binding module (Elia et al., 2003). The PBD recognizes a Ser-[pSer/pThr]-[Pro/X] motif on the substrate (Lowery et al., 2004). Very interesting models exist to propose how the PDB works in the cell cycle. The "processive phosphorylation" model predicts that the Polo PBD first binds to a site on a target protein that has been previously phosphorylated by a priming kinase such as Cdk1/Cyclin B or MAP kinases (these kinases can also phosphorylate the Ser-[pSer/pThr]-[Pro/X] motif). Thus, the Polo kinase domain can phosphorylate other sites in the substrate. The "distributive phosphorylation model" alternatively predicts that the

Polo-box domain binds to a phosphorylated scaffolding or docking protein that helps localize the Polo kinase domain to specific subcellular target substrates (Lowery et al., 2004).

As mentioned above, Polo-like kinases are involved in the Cdk1/Cyclin B-Cdc25 autoamplification loop by phosphorylating and activating Cdc25. In fact, after the initial Polo→Cdc25→Cdk1/Cyclin B activation loop, Polo activity is further enhanced by the Cdk1/Cyclin B-dependent phosphorylation (Abrieu et al., 1998). Moreover, as I also discussed, Polo-like kinases coordinate the nuclear localization and activation of Cdk1/Cyclin B during the G2/M transition to promote nuclear envelope breakdown. A more direct role for Polo in promoting nuclear envelope breakdown has been shown in *C.elegans* where disruption of PLK-1 via RNA-mediated interference (RNAi) results in defects in nuclear envelope breakdown prior to ovulation, a defect that is phenocopied by RNAi-mediated disruption of the *C. elegans* homolog of Cdk1, NCC-1 (Chase et al., 2000).

Polo-like kinases have also been shown to regulate many other processes during mitosis/meiosis including centrosome duplication and spindle formation (Dai et al., 2002; Golsteyn et al., 1995; Lane and Nigg, 1996; Llamazares et al., 1991; Ohkura et al., 1995; Sumara et al., 2004; Sunkel and Glover, 1988), cytokinesis (Bahler et al., 1998; Carmena et al., 1998; Ohkura et al., 1995; Song and Lee, 2001) and activation of the anaphase promoting complex/cyclosome (APC/C) (Charles et al., 1998; Descombes and Nigg, 1998; Golan et al., 2002). One key Polo function is to contribute to the APC/C activation and promote mitotic progression. Plk1 phosphorylation is required to induce the degradation of the APC inhibitor Early Mitotic Inhibitor 1 (Emi1) via the E3 ubiquitin

ligase $SCF^{\beta\text{-}TRCP}$ (Eckerdt and Strebhardt, 2006). This event is necessary to trigger the association of the APC with its activator Cdc20 and the subsequent degradation of Cyclin B (see below) (Eckerdt and Strebhardt, 2006). In addition, Polo can induce the phosphorylation and $SCF^{\beta\text{-}TRCP}$- dependent degradation of Wee1 (Vodermaier, 2004). Interestingly, once Polo performs these roles, it becomes a target of the APC/C itself and undergoes degradation (Eckerdt and Strebhardt, 2006; Lindon and Pines, 2004).

In different organisms, Polo kinase is also critical in the regulation of sister-chromatid cohesion, a process that is fundamental for proper chromosome segregation in mitosis and meiosis (Alexandru et al., 2001; Sumara et al., 2002; Sunkel and Glover, 1988). Cohesion is mediated by the cohesin complex, which is composed of four evolutionarily conserved subunits, two SMC (Structural Maintenance of Chromosome) proteins named Smc1 and Smc3, a kleisin (superfamily of bacterial and eukaryotic SMC protein partners) subunit named Mcd1 (also known as Scc1 and Rad21), and Scc3 (reviewed in (Onn et al., 2008). Homologous chromosomes remain paired until the onset of anaphase I due to cohesion along the arms of sister-chromatids (Miyazaki and Orr-Weaver, 1994). During the metaphase I-anaphase I transition, cohesion on the arms is lost to allow the segregation of the homologs. However, the sister chromatids remained attached by pericentric (heretochromatin) cohesion until their separation during anaphase II (Miyazaki and Orr-Weaver, 1994). In both cell divisions, anaphase I and II are triggered by the cleavage of cohesin by the protease separase (Onn et al., 2008; Uhlmann, 2001). During most of the cell cycle, separase is inactivated by the inhibitory subunit securin (Vodermaier and Peters, 2004). At the onset of anaphase, securins are degraded by the APC/C to release separase (Vodermaier and Peters, 2004).

Chromosomal loss of cohesion occurs sequentially. First, phosphorylation of a cohesin subunit Rec8 by Polo kinase drives the cleavage of cohesin by separase at the chromosome arms between the sister-chromatids to release cohesion (Brar et al., 2006). Phosphorylation of cohesin subunits by Polo kinase enhances their cleavage by separase at anaphase (Alexandru et al., 2001; Sumara et al., 2002; Sunkel and Glover, 1988). Intriguingly, although separase is active, cohesion at the centromere is kept intact. Pioneer studies in flies and yeast found that a "protector" protein prevents the action of separase thus inhibiting cohesin cleavage at the centromeres until anaphase II. This protein was identified as MEI-S332 (*Drosophila*) or Shugoshin (Sgo) (budding and fission yeast) (Clarke et al., 2005; Katis et al., 2004; Kitajima et al., 2004; Marston et al., 2004; Rabitsch et al., 2004). At the metaphase-anaphase transition, phosphorylation of MEI-S332 by Polo kinase results in its release from the centromere concurrently with sister chromatid release by cohesin cleavage (Clarke et al., 2005). Some studies have suggested that the phosphorylation by Polo is antagonized by the phosphatase activity of PP2A to prevent premature cohesion loss (Rivera and Losada, 2006).

The APC/C promotes the metaphase-anaphase transition via inactivation of Cdk1/Cyclin B and degradation of key cell cycle regulators

Ubiquitin-mediated degradation is a major regulatory event in the cell cycle that is achieved by the ubiquitin-proteasome pathway. Ubiquitin (Ub) is attached to a lysine residue of a substrate by a three-step enzyme pathway consisting of a Ub-activating enzyme (E1), a Ub-conjugating enzyme (E2) and a Ub-protein ligase (E3) (Pickart, 2001). Addition of more than one Ub (polyubiquitination chain) to a specific lysine residue (Lys 48) targets proteins to the 26S proteasome for degradation (Pickart, 2001).

The E3-ligase is responsible for the coordination of ubiquitination of target substrates (d'Azzo et al., 2005).

Protein degradation in the cell cycle is mediated by the conserved eleven-subunit anaphase-promoting complex/cyclosome (APC/C) E3 ligase and by its related E3 complex, the SCF (Skp1/cullin/F-box protein) (Note: I am only going to discuss APC functions). The APC/C drives the metaphase-anaphase transition or M-phase exit by promoting degradation of Cyclin B, securins (inhibitors of separase), and other proteins including Polo kinase and Cdc25 (Donzelli et al., 2002; Vodermaier, 2004). Substrate recognition by the APC/C is based on the nine amino acid Destruction-box (D-box) (Glotzer et al., 1991) and the KEN box (defined by the presence of amino acids lysine (K), glutamic acid (E), and asparagine (N)) (Pfleger and Kirschner, 2000). The initial activation of the APC/C largely depends on its phosphorylation and association with the Cdc20/Fizzy activator (Murray, 2004). Although controversial, phosphorylation by Cdk1/Cyclin B is thought to promote both of these events (Murray, 2004). As M-phase exit proceeds, the APC/C associates instead with a second activator, the Cdh1-Fizzy related protein (Murray, 2004). This switch is triggered by the drop in Cdk activity, which normally keeps Cdh1 in an inactive state (Jaspersen et al., 1999; Zachariae et al., 1998). In vertebrates, phosphorylation of the APC inhibitor Emi1 by Plk1 and Cdk1/Cyclin B promotes the SCF-dependent degradation of Emi1 and contributes to the activation of APC/C^{Cdc20} (Vodermaier, 2004). Both activators recognize and bind to target proteins and recruit them to the APC/C core complex for degradation (Hilioti et al., 2001; Pfleger et al., 2001; Schwab et al., 2001). Before anaphase, the APC/C^{Cdc20} targets cyclins and securins for degradation (Sullivan and Morgan, 2007). During late anaphase,

the APC/C^{Cdh1} promotes the degradation of other proteins including Cdc20, Cdc25 and Polo kinase (Sullivan and Morgan, 2007).

Maintenance of prophase I arrest in vertebrate and invertebrate systems

Low Cdk1 activity is required to maintain the prophase I arrest (Choi et al., 1991; Doree and Hunt, 2002; Hong et al., 2003; Jones, 2004; Sugimura and Lilly, 2006) (Figure 1.2B). Prior to fertilization, oocytes arrest twice during meiosis. The first meiotic arrest takes place at prophase I, which can be recognized by an intact germinal vesicle or nuclear envelope. This arrest is highly conserved among species and can last from a few days to many years (e.g. decades in humans) (Figure 1.2A). During this arrest, in addition to undergoing growth and differentiation, the oocyte must remain fully arrested to maintain genome integrity (Kishimoto, 2003; Page and Orr-Weaver, 1997; Sagata, 1996; Whitaker, 1996).

As discussed above, Cdk1/Cyclin B inactivation is controlled at many levels. In mammals and *Xenopus*, cyclic adenosine monophosphate (cAMP) also inactivates Cdk1/Cyclin B. It is known that elevated intracellular cAMP within the oocyte maintains meiotic arrest (Cho et al., 1974; Conti et al., 2002; Conti et al., 1998; Taieb et al., 1997) (Figure 1.2B). This model was first supported by the *in vitro* manipulation and elevation of cAMP levels inside the oocyte by using non-hydrolysable cAMP analogues or phosphodiesterase (PDE) inhibitors and the observed meiotic arrest (Conti et al., 1998;

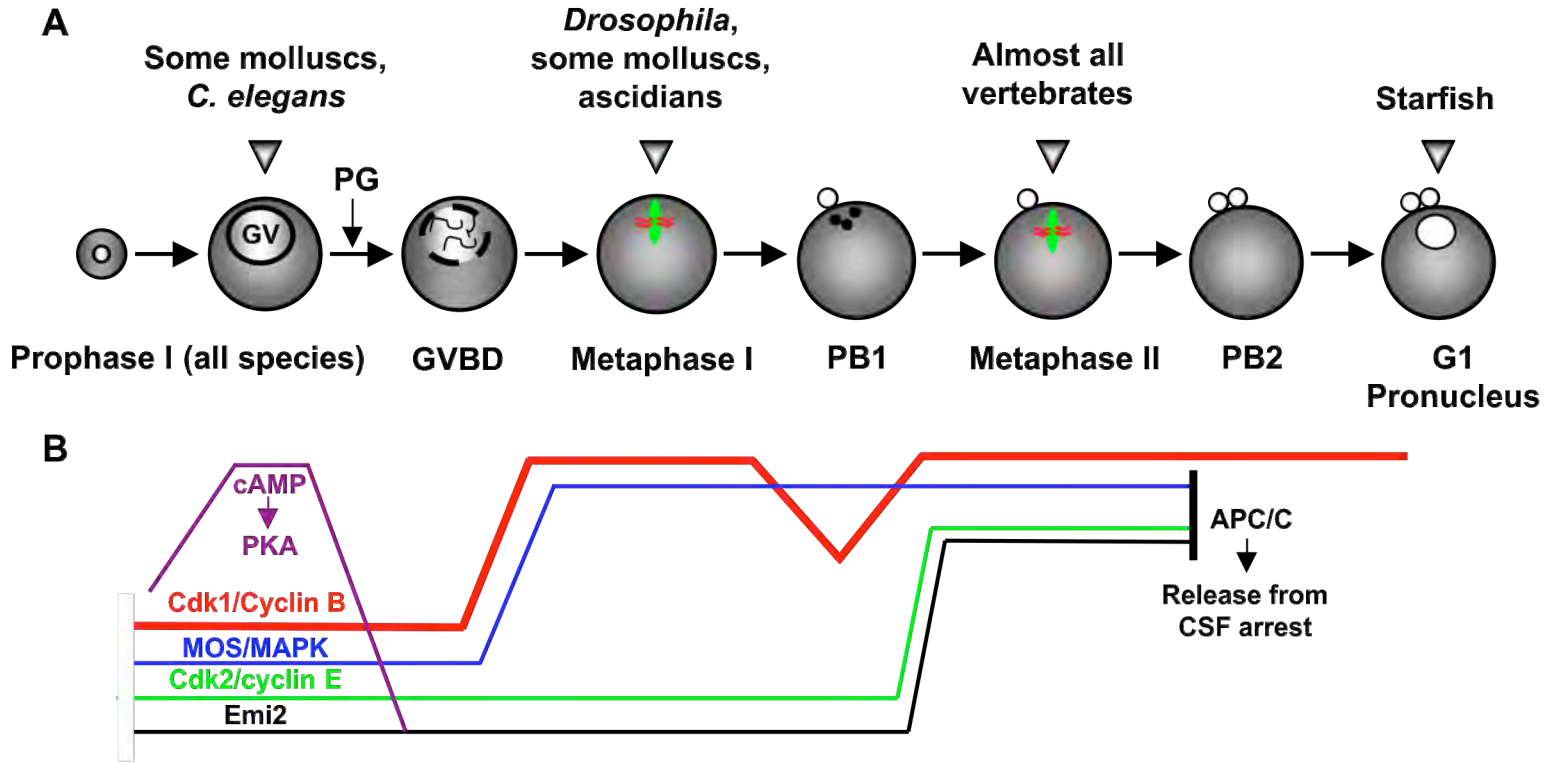

Figure 1.2. Meiosis in oocytes from many species involves strict regulation of Cdk1/Cyclin B activity. (A) During oogenesis, oocytes from most species arrest a first time in prophase I from a few days to many years (humans). Upon hormonal or developmental signals, oocytes undergo meiotic maturation, release the first arrest and depending on the species, enter a second arrest at metaphase I (invertebrates) or metaphase II (many vertebrates). (B) High levels of Cdk1/Cyclin B are required to promote meiotic maturation and for progression into metaphase I or II. In vertebrates, the activity of other signaling pathways (cAMP, MAPK, Emi2) is also known to be regulated in a cell cycle dependent manner.

Tsafriri et al., 1996). Later on, it was suggested, based on new mechanistic evidence from *Xenopus* data, that the high cAMP levels in the oocyte were required to activate protein kinase A (PKA) and most likely inactivate Cdk1/Cyclin B. In addition, PKA activation leads to the phosphorylation and activation of the Wee1/Myt 1 kinases and the Ser 287/216 phosphorylation and inactivation of Cdc25C (Perry and Kornbluth, 2007). Thus, PKA kinase controls Cdk1/Cyclin B activity by promoting the activation of an inhibitor and the repression of an activator. Inhibition of PKA results in exit from the first arrest and resumption of meiosis in the absence of progesterone (Bornslaeger et al., 1986; Maller and Krebs, 1977).

Although the source of the inhibitory cAMP in mammals remains controversial, one hypothesis predicts that the surrounding cumulus granulosa cells produce cAMP that diffuses to the oocyte through gap junctions to maintain the first meiotic arrest (Anderson and Albertini, 1976; Bornslaeger and Schultz, 1985; Dekel, 1988; Eppig, 1991; Webb et al., 2002). A challenging hypothesis establishes that the oocyte produces its own cAMP through a G-Protein-coupled receptor (GPR3) in the oocyte plasma membrane that activates the G protein G_s, which in turn stimulates adenylyl cyclase to increase production of cAMP (Deng et al., 2008; Mehlmann, 2005a; Mehlmann et al., 2002; Mehlmann et al., 2004; Rios-Cardona et al., 2008). Consistent with a role of gap junctions in promoting prophase I arrest and thus inhibiting meiotic maturation, *in vivo* studies by Whitten and Miller (Whitten and Miller, 2007) have shown that, in *C. elegans*, gap junctions transmit a yet unidentified signal from the surrounding somatic sheath cells to the oocyte that maintains the meiotic prophase I arrest in the absence of sperm (the source of the meiotic maturation signal; see below).

In starfish, the immature oocyte has inactive Cdk1/Cyclin B complexes, and it has been suggested that a highly active Wee1-like kinase also inhibits Cdc25 to maintain prophase arrest (Okumura et al., 1996; Picard et al., 1991). Specifically, purified and active Cdk1/Cyclin B kinase injected into arrested starfish oocytes is completely inactivated through the phosphorylation of Thr14 and Tyr-15 residues by the Wee1-like kinase (Kishimoto, 1998). Contrary to the oocytes from most species, which arrest at the diplotene phase of prophase I, the *C. elegans* oocytes arrest at diakinesis of prophase I (McCarter et al., 1999). Nonetheless, Wee1 downregulation is also involved in this arrest. Recent studies have shown that DAF-21, the *C. elegans* homolog of the molecular chaperone heat shock protein 90 (Hsp90), is required for the proper folding and stabilization of Wee1.3/Myt1 thus ensuring the phosphorylation and negative regulation of Cdk1 during the diakinesis arrest (Inoue et al., 2006).

Several molecular events lead to the maintenance of prophase I arrest in *Drosophila*. High levels of *dacapo* (*dap*), a p21CIP/p27KIP1/p57KIP2-like casein kinase I gene, within the oocyte maintain meiotic arrest by inhibiting the kinase activity of Cdk2/Cyclin E and preventing DNA replication or entry into the endocycle (Hong et al., 2003). Consistent with the evolutionarily conserved role of Cdk1/Cyclin B, *Drosophila* oocytes also need to maintain low levels of Cdk1/Cyclin B to maintain a prolonged prophase I arrest. As mentioned above, the APC targets cyclins for degradation (Vodermaier, 2004). Female-sterile mutations in the *morula* gene, which encodes the APC/C subunit APC2 (Kashevsky et al., 2002), result in nurse cell chromosome condensation and entry into a metaphase-like arrest (Reed and Orr-Weaver, 1997). Consistent with the lack of APC function and high Cdk1/Cyclin B activity, *morula*

mutants display inappropriately high levels of Cyclin B protein. Similarly, Reis et al (Reis et al., 2006) determined that mouse oocytes arrested at prophase I have an active APC and that this activation is mediated by one of its two co-activators, Cdh1. They showed that degradation of Cyclin B by APC^{Cdh1} is needed to prevent germinal vesicle breakdown and thus maintain prophase I arrest. Consistent with these findings, depletion of the Emi1 protein, a known inhibitor of the APC, from prophase I-arrested mouse oocytes leads to a delay in progression to metaphase I, and this effect requires Cdh1 (Marangos et al., 2007). In addition, recent data by Lilly and colleagues (Sugimura and Lilly, 2006) have shown that the translational repressor Bruno (Bru), encoded by the *arrest* (*aret*) gene, inhibits the translation/expression of mitotic cyclins (Cyclin A and Cyclin B) after meiotic entry and thus maintains low levels of Cdk1/Cyclin B.

In *C. elegans*, an additional player involved in the maintenance of prophase I arrest is the mitogen-activated protein kinase (MAPK) (MPK-1/SUR-1 in *C. elegans*) phosphatase LIP-1 (Hajnal and Berset, 2002). MAPK expression in *C. elegans* is regulated spatially and temporally during the meiotic cell cycle (Greenstein, 2005). Germ cells that are in pachytene of prophase I require MPK-1/SUR-1 to progress into diakinesis; once they reach this stage, however, MPK-1/SUR-1 is rapidly inactivated (Church et al., 1995; McCarter et al., 1999). LIP-1 is responsible for dephosphorylating and inactivating MPK-1/SUR-1 in the germ cells that exit pachytene to allow the oocyte to arrest at diakinesis (Hajnal and Berset, 2002). Finally, also in *C. elegans*, studies with the Major Sperm Protein/MSP, indicate that, in the absence of sperm, the VAB-1 Eph receptor protein-tyrosine kinase and the POU-class homeoprotein CEH-18 inhibit meiotic maturation (Miller et al., 2003).

Meiotic maturation in vertebrate and invertebrate systems

Although the signaling pathways leading to meiotic maturation and release from prophase I arrest may differ among species, all oocytes must accomplish a universal goal, namely the activation of Cdk1/Cyclin B (Figure 1.2). In *Xenopus*, the steroid hormone progesterone initiates oocyte maturation by decreasing levels of cAMP through binding to its G-protein-coupled receptor (GPCR) and inhibiting adenylyl cyclase activity (Tunquist and Maller, 2003). Activation of the *Xenopus* MAPK signaling pathway has also been shown to lead to phosphorylation and inhibition of Myt1 by Mos (MAPKKK) thus promoting Cdk1/Cyclin B activation and meiotic resumption (Tunquist and Maller, 2003). Similar to sponges, annelids, and mollusks, worms require sperm to undergo meiotic maturation. The MSP signal functions as a hormone that promotes *C. elegans* oocyte maturation and gonadal sheath cell contraction from a distance (Miller et al., 2001). Mechanistically, this is accomplished by the antagonizing activity of MSP on two parallel pathways (i.e. VAB-1 Eph receptor protein-tyrosine kinase and somatic gonadal CEH-18 sheath cell pathways) and the activation of the MAPK pathway (Miller et al., 2001; Miller et al., 2003). Because sperm triggers meiotic maturation in this system, oocytes complete meiosis without a second arrest. Interestingly, MAPK activation within the oocyte does not seem to be required for meiotic maturation in mammals (Liang et al., 2007), and Mos function is dispensable for *Drosophila* meiotic maturation (Ivanovska et al., 2004).

In mammals, a surge of luteinizing hormone (LH) from the pituitary gland right before ovulation promotes resumption of meiosis. Although unclear, the current hypothesis predicts that LH promotes meiotic maturation potentially via inhibition of

GPR3 or G_s (which promote the synthesis of cAMP), Ca^{2+} (shown to inhibit mouse adenylyl cyclase) or, alternatively, via the activation of the oocyte PDE3A (which hydrolyzes and decreases cAMP) (Mehlmann, 2005a; Mehlmann, 2005b). In both mammals and *Xenopus* systems, the resulting decrease in cAMP leads to inactivation of PKA and release of Cdc25 inhibition. In starfish, 1-methyladenine (1-MeAde) serves as a maturation-inducing hormone by inducing a putative kinase that phosphorylates and activates Cdc25. In many organisms, the AKT/PKB pathway also inhibits the Myt1-like kinase to activate Cdk1/Cyclin B in the cytoplasm (Okumura et al., 2002). Feedback control from activated Cdk1/Cyclin B and activation of Cdc25 by Polo kinase (Plk) further activate Cdk1/Cyclin B (Kanatani et al., 1969; Kishimoto, 1999). Interestingly, novel activators of Cdk1/Cyclin B have been discovered in vertebrate oocytes including *Xenopus*, humans and mouse (Gastwirt et al., 2007). Similar to Cyclin B, members of the Speedy/RINGO family bind to and activate Cdk1 to induce meiotic maturation (Ferby et al., 1999; Terret et al., 2001). No homologs have been identified in invertebrates.

Our knowledge of the process of meiotic maturation in *Drosophila* is very limited. The signal that triggers oocyte meiotic maturation has not been identified. However, based on recent findings, prostaglanding hormones could serve as the stimulatory signal. *Drosophila* cyclooxygenase (COX), which is one of the enzymes responsible for synthesis of prostaglandins, promotes early ovarian follicle maturation (Tootle and Spradling, 2008). Thus, COX could be inducing meiotic maturation at later stages in a similar fashion.

In all the systems described above, Cdk1/Cyclin B activation triggers meiotic maturation by promoting the phosphorylation of factors involved in germinal vesicle or

nuclear envelope breakdown, chromosome condensation, and spindle assembly. These events are absolutely required for progression into and establishment of the second arrest in meiosis (reviewed in (Jones, 2004; Kishimoto, 2003).

Maintenance of the second meiotic arrest in vertebrate and invertebrate oocytes

Following nuclear envelope breakdown, oocytes will progress through meiosis and undergo a second arrest at metaphase I (many invertebrates) or metaphase II (most vertebrates including humans). During this second arrest, Cdk1/Cyclin B activity reaches its maximum levels. In vertebrates, Cdk1/Cyclin B stability depends on the Cytostatic Factor (CSF), hence the "CSF arrest" name (Figure 1.2). Pioneering work by Masui and Markert (Masui and Markert, 1971) identified CSF as an activity in the cytoplasm of mature oocytes that induced metaphase arrest when injected into two-cell embryos. To date, three pathways are known to contribute to the establishment and/or maintenance of CSF arrest; MOS/MEK1/MAPK/$p90^{Rsk}$, Emi2 and the Cdk2/Cyclin E pathways (Tunquist and Maller, 2003). Although their roles remain to be fully elucidated, it is thought that all of these pathways function to ultimately inhibit the activity of the APC/C (Tunquist and Maller, 2003) (Figure 1.2B), which, as mentioned before, is required to promote anaphase.

Drosophila oocytes arrest at metaphase I during stage 13 in oogenesis (more details described below) (King, 1970). The current hypothesis for the maintenance of metaphase I arrest in flies is that the tension resulting from the exchange events between the homologs (by chiasmata) sends a signal to maintain the metaphase I arrest (McKim et al., 1993). Based on the failure of *cdc2* (encodes Cdk1) or *twine*^{1} (*Cdc25* homolog)

mutants to arrest in metaphase I (as described in Chapter II), *Drosophila* oocytes seem to also require high levels of Cdk1/Cyclin B activity to establish and maintain this second arrest. In contrast, simpler invertebrate oocytes do not arrest at either metaphase I or II and complete meiosis in a fertilization-dependent or -independent manner. For example, once prophase I arrest is released, starfish oocytes proceed to complete meiosis II, uninterruptedly, and enter mitosis. If not fertilized, the oocyte arrests for a second time at the female pronucleus stage (G_1 phase) (Kishimoto, 1998) (Figure 1.2A). During this arrest, MAPK activity remains high but Cyclins A and B remain low (Kishimoto, 1998).

The *Drosophila* ovary, a model system to study female meiosis

Ovary description

As described above, the molecular composition and regulation of the cell cycle are remarkably conserved. The *Drosophila* ovary has been instrumental in the understanding of the regulation of the meiotic cell cycle. This system offers powerful genetic and molecular tools that are useful to investigate the cell cycle machinery.

Each *Drosophila* ovary is composed of fifteen to twenty ovarioles, which contain progressively older egg chambers (Spradling, 1993a). At the anterior-most tip of each ovariole, the germarium houses germline (GSC), somatic (SSC) and escort (ESC) stem cells. GSCs divide asymmetrically and give rise to cystoblasts, which undergo four rounds of division with incomplete cytokinesis to form a 16-cell germline cyst interconnected by ring canals (Figure 1.3) (Spradling, 1993b; Spradling et al., 1997). Every cyst leaves the germarium as an egg chamber surrounded by a monolayer of mitotically active somatic follicle cells.

Oocyte meiotic entry

In *Drosophila*, entry into meiosis begins in region 2A of the germarium (Figure 1.4B). The oocyte fate is gradually restricted to one cell in the cyst. Initially, the two cells within the germline cyst containing four ring canals (Figure 1.4A) (called the "pro-oocytes") enter meiosis and form the synaptonemal complex (where homologous chromosomes synapse and undergo exchange) (Carpenter, 1975; King, 1970). As the pro-oocytes progress into pachytene of prophase, the two cells with three ring canals also form a zygotene-like synaptonemal complex and enter meiosis (Huynh and St Johnston, 2000). Soon afterwards, however, in the middle of region 2A, the synaptonemal complex disappears from the cells containing three ring canals but remains in the two pro-oocytes. The two pro-oocytes containing synaptonemal complex also accumulate oocyte determinants such as Egl, BicD, and Orb (Huynh and St Johnston, 2000; Navarro et al., 2001). Through a yet unknown mechanism, by region 2B, the oocyte determinants and the synaptonemal complex only concentrate in one of the two pro-oocytes (Figure 1.4B) whereas the second pro-oocyte loses its synaptonemal complex and exits meiosis. The one pro-oocyte containing oocyte determinants, synaptonemal complex and the microtubule organizing center (MTOC) becomes the oocyte (Figure1.4B). The microtubule network is then extended to the remaining fifteen cells in the cyst, which become nourishing nurse cells (Navarro et al., 2001). As the egg chamber leaves the germarium in region 3, the oocyte locates to the posterior end and the oocyte DNA compacts into a karyosome (see Figure 1.5A) (Huynh and St Johnston, 2000). The synaptonemal complex persists during the early stages of oogenesis but eventually disappears (around stage 6) (Hawley et al., 1993). The oocyte and the nurse cells have

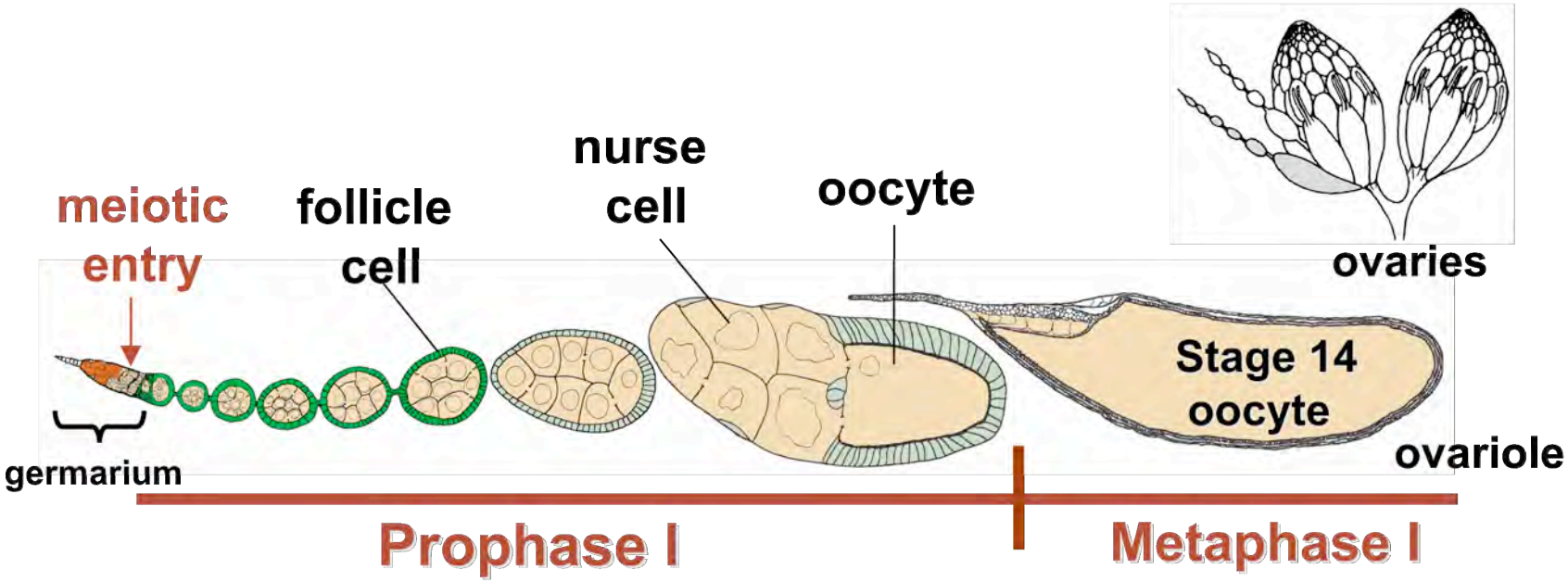

Figure 1.3. The *Drosophila* ovary. *Drosophila* females contain a pair of ovaries that are composed of ovarioles containing progressively older egg chambers. At the tip of each ovariole, the germarium houses three populations of stem cells that are required to produce each egg chamber containing somatic and germline components. The oocyte enters meiosis in the germarium and remains arrested at prophase I until the onset of meiotic maturation at stage 13. Stage 14 oocytes are arrested in metaphase I and also undergo dehydration.

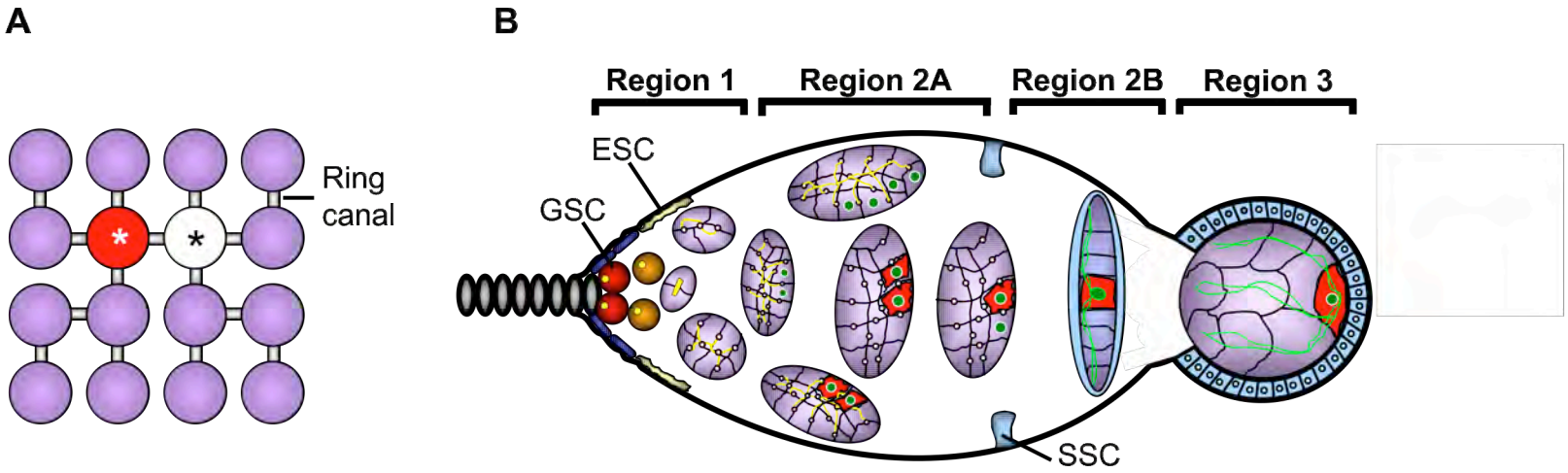

Figure 1.4. *Drosophila* oocyte determination and entry into meiosis. (A) All sixteen cells within a germline cyst are interconnected by actin-rich ring canals. Asterisks point to pro-oocytes surrounded by four ring canals. Red circle, future oocyte; white circle, pro-oocyte that exits meiosis. (B) Germarium divided into three regions. **Region 1**: Germline stem cells give rise to a cystoblast that divides 4 times with incomplete cytokinesis to form a 16-cell cyst. Escort stem cells give rise to escort cells, which envelope the cystoblast and the 2-, 4-, 8-, and early 16- cell cysts. **Region 2A**: The two cells within the cyst containing four ring canals enter meiosis and form the synaptonemal complex (SC) (green circles). The surrounding cells containing three ring canals also enter meiosis and form SCs that soon after disappear. Only the two pro-oocytes retain the SC and accumulate oocyte determinants (Egl, BicD, Orb) (red), which are restricted to the future oocyte. **Region 2B**: Only one of the two pro-oocytes contains SC, oocyte determinants, centrioles and MTOC (light green) and remains in meiosis. The second pro-oocyte eventually loses the SC and reverts to the nurse cell pathway along with the rest of the cells in the cyst. **Region 3**: The oocyte locates at the posterior of the egg chamber along with all its components. The egg chamber compacts its DNA into a karyosome and the oocyte retains the SC until stage 6 of oogenesis. GSC, germline stem cell; ESC, escort stem cell; SSC, somatic stem cell. Adapted from Navarro et al. 2001.

distinct cell cycles. The nurse cells enter the endocycle (endoreplicative cycle where cells undergo successive rounds of DNA replication without an intervening mitosis) to become highly polyploid (Hong et al., 2003), whereas the oocyte arrests at pachytene of prophase I during most of its growth and development (Figure 1.3) (King, 1970).

Chromosome behavior during prophase I and metaphase I in *Drosophila*

Oocytes in *Drosophila* do not go through the diplotene-diakinesis phases of prophase I (Hawley et al., 1993). Instead, after arrest at pachytene, the chromosomes condense tightly into a karyosome (Figure 1.5A) (King, 1970) and remain in this state until metaphase I at stage 14. Drosophila has four pairs of chromosomes: the sex chromosomes X/Y and the autosomes 2, 3, and 4. After meiotic maturation and nuclear envelope breakdown, the oocyte progresses into metaphase I arrest. The chromosomes themselves assemble the spindle due to the lack of centrosomes in the oocyte (Theurkauf and Hawley, 1992). Once in metaphase I, all chromosomes align on the bipolar spindle with the two non-exchange fourth chromosomes positioned between the metaphase plate and the poles, and the exchange chromosomes tightly associate via chiasmata at the metaphase plate (Theurkauf and Hawley, 1992) (Figure 1.5B).

In cooperation with the bipolar spindle and associated motor proteins and sister chromatid cohesion, the chiasmata resulting from the early prophase I crossing over events direct the proper segregation of the autosomes 2, 3, and X during the metaphase I-anaphase I transition (McKim et al., 2002). In contrast, the fourth chromosomes do not undergo exchange and are therefore obligated achiasmate. Even in the absence of chiasmata, these chromosomes can segregate faithfully after the first meiotic division

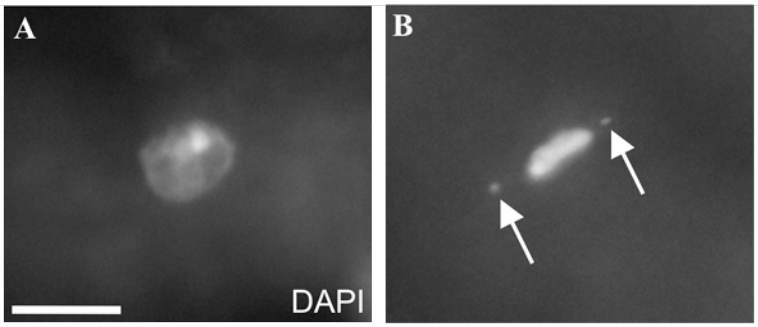

Figure 1.5. The stages of *Drosophila* meiosis can be recognized based on DNA morphology. During prophase I, the *Drosophila* oocyte nucleus is condensed into a karyosome (A), which has a bright spot corresponding to heterochromatin. This karyosome is disassembled when the chromosomes condense further and align at the metaphase plate in metaphase I (B). The fourth non-exchange chromosomes (arrows) move and position themselves toward opposite poles. The exchange chromosomes remain associated in the center via chiasmata. Scale bar, 10 μm.

(Grell, 1963; Hawley et al., 1993). This is accomplished by a “backup” mechanism based on the alignment and pairing via heterochromatic homology between the homologs that begins during early prophase but persists until metaphase I, thus maintaining the physical association of the achiasmate chromosomes (Dernburg et al., 1996; Hawley et al., 1992; Karpen et al., 1996). In fact, in contrast to the autosomes where chiasmata ensure the orientation of the centromeres, heterochromatin pairing is required to properly orient the centromeres at metaphase I and thus ensure their proper segregation (Hawley et al., 1992; Karpen et al., 1996).

In addition to arrest at metaphase I, oocytes undergo dehydration at stage 14, a process that is reversed during the passage of the oocyte through the oviduct by the re-absorption of water, which is thought to promote egg activation and completion of meiosis (Mahowald et al., 1983).

Meiotic maturation is not well understood in *Drosophila*

Although we have obtained some insights regarding the regulation of prophase I in *Drosophila,* the developmental and molecular events controlling the timing of meiotic maturation and progression into metaphase I are not well understood. Very few genes have been described as being involved in these processes. As mentioned above, high levels of Cdk1/Cyclin B are required to promote maturation and second arrest at meiosis I. For example, mutants of *twine,* the meiotic homolog of the Cdc25 phosphatase, fail to progress into metaphase I (Alphey et al., 1992; Courtot et al., 1992; White-Cooper et al., 1993), underscoring the potential requirement of activated Cdk1 in flies. The timing of meiotic maturation has been shown to be altered in *Matrimony* (*Mtrm*) mutants (Xiang et

al., 2007). The current model predicts that nuclear envelope breakdown is controlled by the Mtrm-induced inhibition of Polo kinase before stage 13 of oogenesis. Independent recent reports by the Glover group identified *greatwall (gwl)* as a conserved protein kinase that, when mutated, leads to failure to arrest in metaphase I due to premature loss of sister chromatid cohesion (Archambault et al., 2007). It has been hypothesized that this defect is caused by lack of Polo inhibition by Gwl (Alexandru et al., 2001).

Despite how little is known about meiotic maturation and metaphase I arrest in *Drosophila*, some of the molecular players that promote anaphase progression during meiosis I and II have been identified. The *cortex* (*cort*) gene, which encodes a distant member of the Cdc20(Fzy)/Cdh1-related protein family, functions together with the APC adaptor Fzy to target Cyclins A, B and B3 for degradation by the APC/C and thus to promote the completion of meiosis I and II (Pesin and Orr-Weaver, 2007; Swan and Schupbach, 2007). In addition, the *Drosophila cyclin-dependent kinase subunit 30A* gene, *cks30A*, has been shown to serve as an activator of both APC^{CORT} and APC^{FZY} during cyclin degradation (Swan et al., 2005; Swan and Schupbach, 2007). It seems, however, that this effect is more specific towards Cyclins A and B3 and that *cks30A* activity is dispensable for some of the functions of APC^{CORT} and APC^{FZY}.

Mammalian *α-endosulfine* has been linked to the physiology of the β-cell in the pancreas and to the control of insulin secretion

Because we have discovered a role for the *Drosophila* homolog of α-endosulfine in meiotic maturation, I will next describe what is known about α-endosulfines.

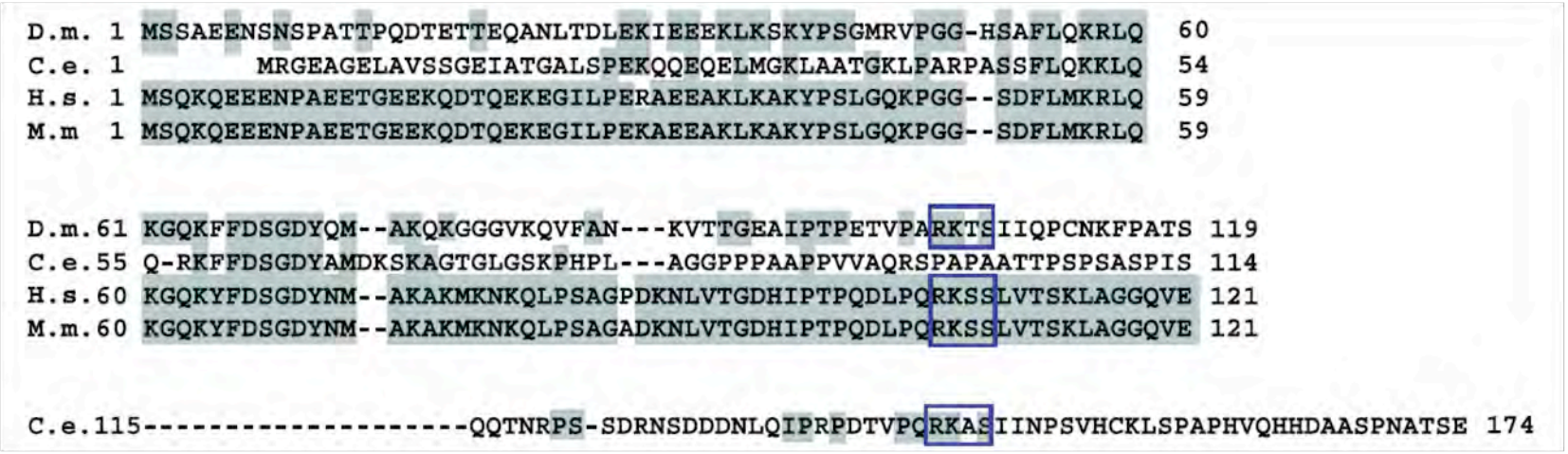

Figure 1.6. *endos* is the *Drosophila* homolog of mammalian α-endosulfine. Fly (D.m) *α-endosulfine* shares 45-47% identity with its human (H.m) and mouse (M.m) counterparts. It also has *C. elegans* (C.e) and yeast (not shown) homologs. A predicted cAMP-dependent phosphorylation site (blue boxes) is highly conserved.

Alpha-endosulfine is a highly conserved 121-amino acid protein that belongs to a cAMP-regulated phosphoprotein family (Figure 1.6) (Peyrollier et al., 1996). This family was initially identified in mammalian brain as a group of neuronal phosphoproteins that were *in vitro* substrates of PKA (Walaas et al., 1983). The human *α-endosulfine* mRNA is found in a wide range of tissues including brain, heart, lung, kidney, pancreas, and liver (Heron et al., 1998), suggesting that it may have multiple biological roles.

α-Endosulfine peptide was isolated and purified from porcine brain extracts based on its ability to compete with [^{3}H]glibenclamide (a potent antidiabetic sulfonylurea drug) for binding to the sulfonylurea receptor (SUR1), an ATP-dependent potassium channel (K_{ATP}) regulatory subunit, in pancreatic *β*-cell membranes (Peyrollier et al., 1996; Virsolvy-Vergine et al., 1992). Although controversial, *in vitro* studies have suggested that α-endosulfine modulates insulin secretion by binding and altering ion channel activity.

α-Endosulfine binds and modulates the pancreatic *β*-cell K_{ATP} channel in cultured mammalian cells

It is well established that pancreatic *β*-cell ATP-dependent potassium channels (K_{ATP}) play an essential role in glucose-induced insulin release by coupling cellular metabolism with plasma membrane excitability. The pancreatic *β*-cell K_{ATP} channel is composed of four inward rectifying subunits (Kir6.2) (Inagaki et al., 1995), which form the channel central pore, and four regulatory Sulfonylurea Receptor subunits (SUR1) that surround the pore (Figure 1.7B) (Aguilar-Bryan et al., 1998; Ashcroft, 2000). The SUR1 protein, which contains seventeen transmembrane domains, belongs to the ATP-Binding

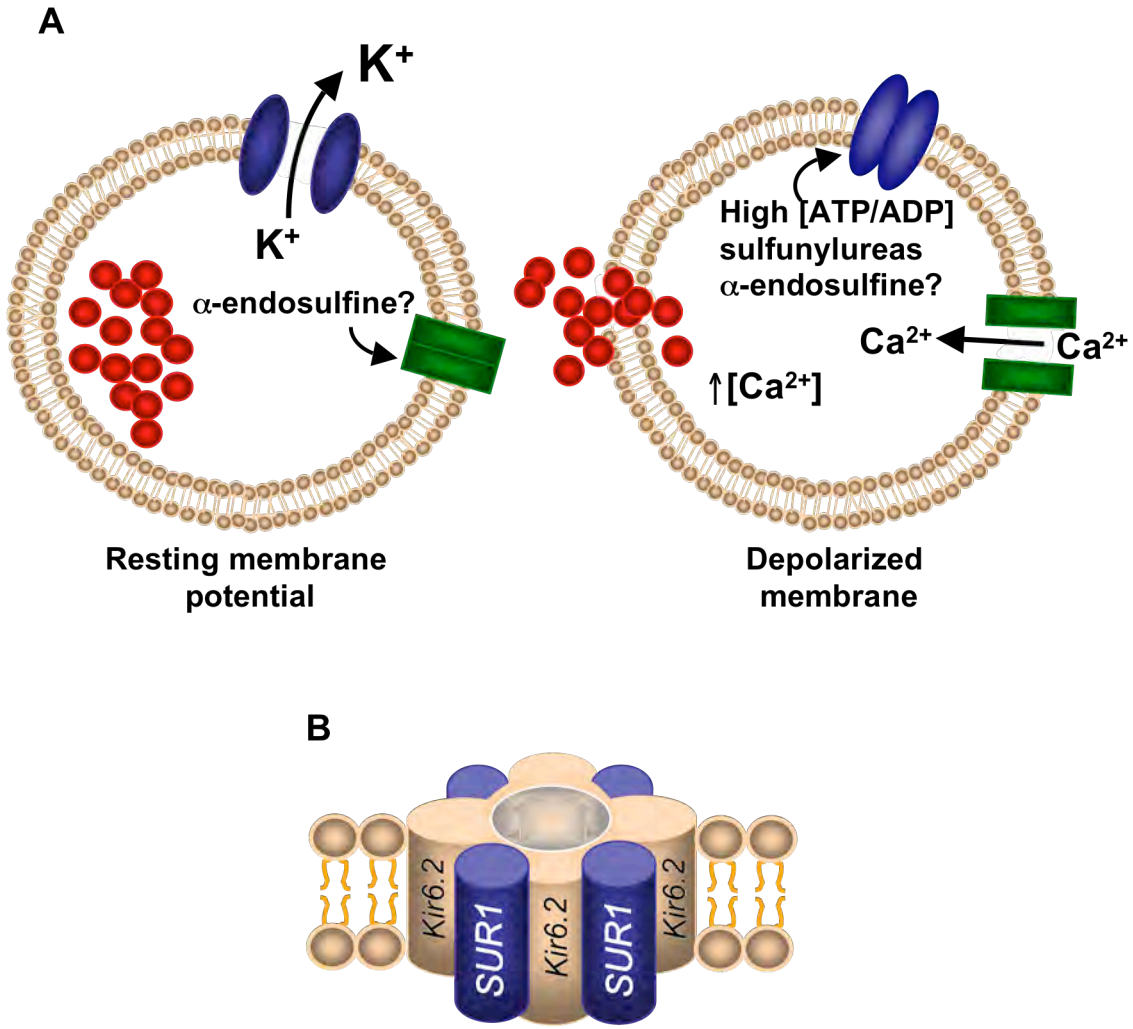

Figure 1.7. *α-endosulfine* has been proposed to induce insulin secretion through modulation of ATP-dependent potassium (K_{ATP}) channels in pancreatic *β*-cells. (A) In mammals, the pancreatic *β*-cell resting potential is determined by K_{ATP} channels (blue) that are normally in an open state. High [ATP]/[ADP] ratio and binding of sulfonylurea drugs to the pancreatic K_{ATP} channel induce channel closure, membrane depolarization, opening of Ca^{2+} channels (green) and secretion of insulin granules (red). It has been proposed that *α -endosulfine* is the endogenous counterpart of sulfonylureas. Other studies argue that, instead, *α-endosulfine* binds to Ca^{2+} channels to prevent the secretion of insulin. (B) Schematic of the pancreatic K_{ATP} channel composed of four *Kir6.2* pore-forming subunits and four regulatory *SUR1* subunits.

Cassette (ABC) family (Aguilar-Bryan et al., 1995). In addition to the β-cell K_{ATP} channel, other Kir6 and SUR isoforms assemble into functional channels in heart and smooth muscle tissues (Burke et al., 2008). Mutations in either the Kir.2 or SUR1 subunits cause Persistent Hyperinsulinemic Hypoglycemia of Infancy (PHHI), an autosomal recessive disorder characterized by irregular insulin secretion due to a failure of K_{ATP} channels to open (Dunne et al., 1997; Kane et al., 1996; Nestorowicz et al., 1997; Nestorowicz et al., 1996).

Opening of K_{ATP} channels sets the resting β-cell membrane potential (-70 mV) whereas their closure depolarizes the plasma membrane (Aguilar-Bryan et al., 1998; Ashcroft and Gribble, 1998; Babenko et al., 1998). An increased rate of glucose metabolism causes an increased [ATP]/[ADP] ratio that results in closure of the K_{ATP} channel (Figure 1.7A). Channel closure induces membrane depolarization that leads to the opening and activation of Ca^{2+} channels, Ca^{2+} entry into the cell and the subsequent secretion of insulin. Other agents such as sulfonylureas (antidiabetic drugs) bind to the regulatory.subunit SUR1 of the K_{ATP} channel, depolarize the β-cell membrane and subsequently stimulate insulin release (Ashcroft et al., 1993).

Evidence from mammalian *in vitro* studies has revealed that like sulfonylureas, α-endosulfine binds to SUR1, inducing channel closure, membrane depolarization and release of insulin (Figure 1.7A). Recombinant human α-endosulfine, when applied either intra- or extracellularly, inhibits the binding of sulfonylureas to their receptors and reduces K_{ATP} channel currents in *Xenopus* oocytes injected with Kir6.2 and Sur (Heron et al., 1998). It also stimulates basal insulin secretion in MIN6 pancreatic β cells. Conflicting *in vitro* studies using perfused rat pancreas have reported, however, that

under glucose-stimulated conditions, α-endosulfine inhibits insulin secretion by binding to Ca^{2+} channels and blocking Ca^{2+} entry into the cell (Virsolvy et al., 2002). Unfortunately, no *in vivo* analyses have been performed to discern between these two models.

The *Drosophila* genome contains homologs for Kir6, SUR1 and α-endosulfine families

Endos is 46% identical and 62% similar to mammalian α-endosulfine. It also shares a putative cAMP-dependent phosphorylation site with human, mouse, worm, and yeast homologues (see Figure 1.6) (Dulubova et al., 2001). Endos is expressed throughout development (Drummond-Barbosa and Spradling, unpublished). In adults, Endos protein is expressed in the germline and somatic cells of the ovary, and from stage 6 of oogenesis it becomes enriched at the cortex of egg chambers (Figure 1.8) (Drummond-Barbosa and Spradling, 2004). It is also expressed throughout the brain and parts of the intestine including the cardia, hindgut, and Malpighian tubules.

Studies by Drummond-Barbosa and Spradling (Drummond-Barbosa and Spradling, 2004) identified two major phenotypes in *endos* mutants: (1) failure to adjust proliferation rates in response to a rich diet and (2) failure to undergo oocyte dehydration at stage 14. On a protein-rich diet, *endos* females showed a 2- to 3-fold reduction in the rate of egg laying. Similar to the phenotype of *chico* mutants (Figure 1.9B) in which the insulin pathway is disrupted, $endos^{00003}$ follicle cells (Figure 1.9D) were not able to fully upregulate their division rates on a rich food source when compared to wild-type follicle cells (Figure 1.9C). On a poor food source, control heterozygous females and $endos^{00003}$

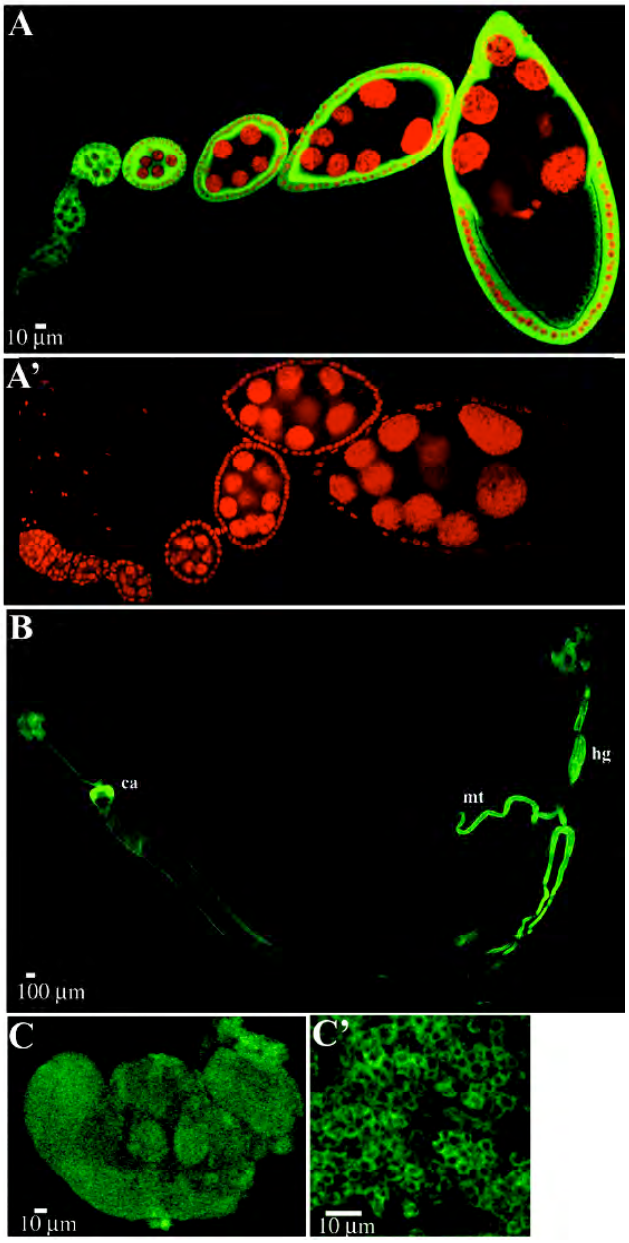

Figure 1.8. Endos expression is found in adult ovary, brain and gut tissues. Endos protein (green) is expressed at high levels in germline and somatic cells of the ovary (A), in the gut (B), and throughout the brain (C). Closer view of pannel C (C'). DNA is labeled with propidium iodide (red). Ca, cardia; mt, Malphigian tubules; hg, hingut. From Drummond-Barbosa and Spradling, 2004.

females showed very similar rates of proliferation. These results, together with genetic mosaic analysis experiments that demonstrated that *endos* is non-cell autonomously required for normal rates of egg chamber development and follicle cell proliferation (Drummond-Barbosa and Spradling, 2004), suggested that *endos* was acting via a secreted factor. In addition, these studies showed that *endos* is cell autonomously required to promote normal stage 14 oocyte dehydration.

Three genes in the *Drosophila* genome (*Dir*, *irk2* and *irk3*) encode putative inward rectifier potassium channels and are expressed in the Malpighian tubules (Evans et al., 2005). *Irk3* mRNA expression has also been detected in the germline (nurse cells and oocytes), whereas *irk2* mRNA has been found to be abundant in the spermatheca (Evans et al., 2005). Based on conservation of specific domains, the *Drosophila* inward rectifier (*Dir*) gene is related to the mammalian Kir6.2 subfamily (MacLean et al., 2002). *Dir* mRNA expression can be found in all stages of embryogenesis, with enrichment in the embryonic salivary glands, and in the larval central nervous system and imaginal discs (MacLean et al., 2002). Functional roles for *Dir*, *irk2* or *irk3* have not been yet directly established in *Drosophila*. *Drosophila Sur* (*Sur*) is the only fly homolog of the vertebrate *SUR1* gene, and it shares high conservation in functional domains with mammalian SUR (77% similarity and 51% identity) (Nasonkin et al., 1999). *Sur* embryonic expression has been detected in the embryonic developing trachea and dorsal vessel ("heart") (Nasonkin et al., 2002). When injected in *Xenopus* oocytes, *Sur* can generate K_{ATP} currents suggesting that it is sufficient to induce channel activity (Nasonkin et al., 1999). Additional roles in chitin synthesis and cardiac development have been recently reported for *Sur* (Abo-Elghar et al., 2004; Hendren et al., 2007).

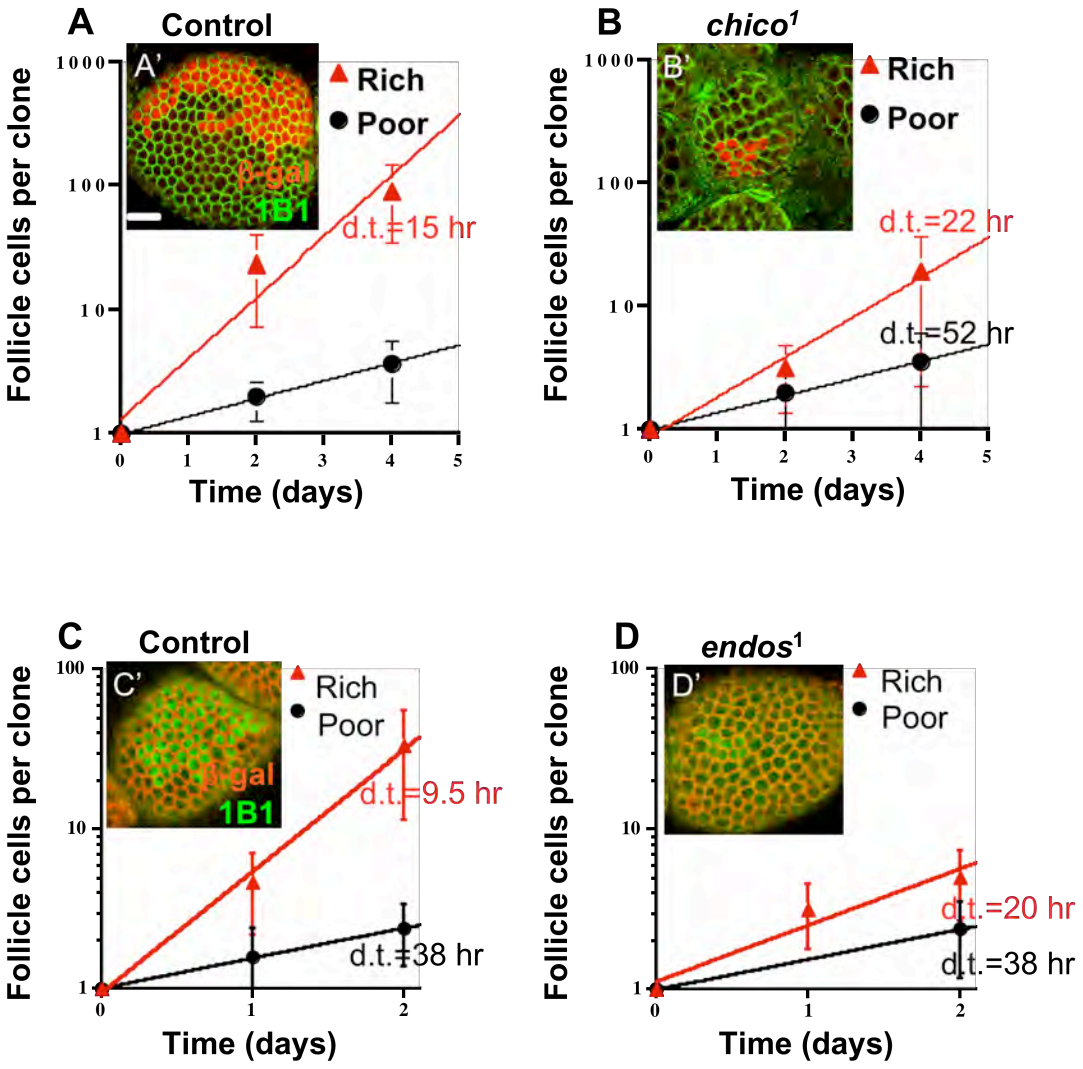

Figure 1.9. The insulin pathway and *endos* mediate the ovarian response to diet. In control females (A and C) kept on a protein-rich diet, follicle cells divide 3- to 4-fold faster than in *chico* (a major insulin pathway component) mutant females (B). β-galactosidase (β-gal, red) labeled clones 4 days after heat shock or 2 days after heat shock are shown in (A',B') and (B',C'), respectively. Each scale bar represents 10 μm. 1B1 (green) labels cell membranes. Similar to *chico*^{1} mutants, *endos*^{1} mutant follicle cells fail to upregulate their division rates in response to a protein-rich diet when compared to control follicle cells (D). Adapted from Drummond-Barbosa and Spradling, *Dev. Biol.*, 2001 (panels A and B) and Drummond-Barbosa and Spradling, *Dev. Biol.*, 2004) (panels C and D).

Based on the proposed link between α-endosulfine and insulin secretion in mammals and our *endos* mutant data, I first tested for a positive role of *endos* in insulin secretion. Our current evidence suggests that, in *Drosophila*, insulin secretion is not affected. We have uncovered, instead, a key role of *endos* in female meiosis and a potential role in mitosis. This dissertation seeks to add new insights into the limited knowledge regarding the developmental and molecular mechanisms underlying the timing of meiotic maturation and progression into metaphase I in *Drosophila* oogenesis. In Chapter II, I describe a detailed analysis of the role of *endos* in meiotic maturation. In addition, I describe the biochemical approach used to identify a novel Dendos interactor, Early girl (Elgi), a predicted E3 ubiquitin ligase, as well as the role of *elgi* in meiosis. Finally, I describe the evolutionary conservation of the molecular function of α-endosulfine. In Chapter III, I present genetic interactions and mutational analysis data that provide insights into the mechanism of action and regulation of *endos* during meiosis. Chapter IV presents evidence that suggests, in contrast to mammalian models, that *endos* is not required for insulin secretion in *Drosophila*. In the final chapter (Chapter V), I summarize the interpretations of my results, future directions and my working model to explain how *endos* might control the cell cycle.

CHAPTER II

α-ENDOSULFINE IS A CONSERVED PROTEIN REQUIRED FOR OOCYTE MEIOTIC MATURATION IN *DROSOPHILA*

This chapter was published in *Development* (Jessica R. Von Stetina, Susanne Tranguch, Sudhansu K. Dey, Laura A. Lee, Byeong Cha and Daniela Drummond-Barbosa. *Development.* 2008; 135(22): 3697-706), and reproduced here almost verbatim, except for additional details added to Materials and Methods section.

The only data that were not obtained directly by me are the mouse immunohistochemistry results. In addition, the pools of ^{35}S-labeled proteins used in the primary DIVEC screen were provided by Drs. Ethan and Laura Lee.

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, while 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, 1993a). 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 completion of meiosis (Mahowald et al., 1983).

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. Cdk1/Cyclin B 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 Cyclins A and B (Sugimura and Lilly, 2006). High levels of Dacapo, a Cdk1 inhibitor, within the oocyte likely 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 results in premature transition from prophase I to metaphase I, although it does 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 via inhibition of Elgi to promote meiotic maturation. Remarkably, germline-specific expression of ENSA, the human α-endosulfine, rescues the *endos* meiotic defect and α-endosulfine is expressed in mouse oocytes, suggesting conservation of the meiotic function of α-endosulfine.

Materials and Methods

Drosophila strains and culture

Fly stocks were maintained at 22-25ºC on standard medium. *yw* was used as 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 29°C incubation 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., 1997; 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 *pUASp-endos* construct was made by amplifying the *dendos* open reading frame plus 21 base pairs of the upstream sequence from the LD19034 cDNA using primers 5'-AGTACTATCACCCGTAGGCAGCACACAATGAGCTCCGCGGAAGAAAACAGCAACAGCCC-3' and 5'-GGATCCTTAGCTCGTCGCCGGGAACTTGTTACAGG-3'. The PCR product cloned into pCR2.1 TOPO (Stratagene) was sequenced, and the insert was excised by *EcoRI* digestion and subcloned into the *UASpI* vector (modified from *pUASp* vector by T. Murphy). Similarly, the human α-endosulfine open reading frame was amplified from EST #5105571 with a 21 base pair sequence added from the region immediately upstream of the *dendos* open reading frame using primers 5'-AGTACTATCACCCGTAGGCAGCACACAATGTCCCAGAAACAAGAAGAAGAGAACCCTGCGG-3' and 5'-GGATCCTCATTCAACTTGGCCACCCGCAAGCTTGC-3', and subcloned into pCR2.1 TOPO. The sequenced insert was subcloned into the pUASpI vector using *ScaI* and *BamHI* to generate *pUASp-hendos.*

To generate the *UASp-myc::twe* construct, the *twine* coding region was amplified from the LD19391 cDNA using primers 5'-GCGGCCGGCCGATGGCGAGCAAGCGGCTAATG-3' and 5'-GCGGCGCGCCTCACTCGGCGTAGAGCAGTCG-3' and subcloned into pCR2.1

TOPO. The *twine* cDNA was excised from the TOPO vector using *FseI* and *AscI* enzymes and subcloned into *pcs2* (modified *UASp* vector containing *FseI* and *AscI* restriction sites and the c-MYC tag), in frame, after the c-MYC tag to generate *UASp-myc::twe*. To generate the *hs-twe* construct, the *twine* cDNA was excised from the TOPO vector with *NotI* and *SpeI* enzymes and subcloned into the *pCasper-hs* vector linearized with *NotI* and *XbaI* enzymes.

Transgenic lines *UASpI-dendos #1/CyO, UASpI-dendos #10/TM3,Sb^{e}, UASpI-Hendos #28/CyO, UASpI-Hendos #54/CyO, UASp-myc::twe #8/FM7c, UASp-myc::twe #11/TM3,Sb^{e} and hs-twe #7/CyO* were generated by germline transformation as described (Spradling and Rubin, 1982).

RNA and protein expression 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), 1:1,000 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), 1:1,000 mouse monoclonal anti-c-Myc (9E10, Sigma), or 1:1,000 mouse monoclonal anti-Cdk1 (anti-PSTAIR, Sigma) antibodies. Alexa 680-conjugated goat anti-rabbit and anti-mouse (Molecular Probes) and IRDye 800-conjugated goat anti-

guinea pig (Rockland) secondary antibodies were used at 1:5,000 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 Byosystems) for priming and SuperScript^{TM} 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 ml of extract with 0.5 ml of anti-Cdk1 (Gawlinski et al., 2007) or 2.5 ml of anti-Cyclin B antibodies, immunocomplexes were isolated using proteinA- or proteinG-sepharose beads. Washed beads were incubated with 16 ml of kinase buffer, 3 mM of histone H1.2, and 2 mCi [^{32}P] ATP for 10-20 minutes at 25^{o}C. Detection and quantification were performed using a Typhoon 9200 Imager and ImageQuant 5.2. Parallel immunoprecipitations using 100 ml of extract and 5 ml of rabbit polyclonal anti-Cdk1 or 25 ml 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, 1993a), 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 Chi-square test.

For visualization of microtubules, ovaries were dissected and teased apart in Robb's media (55mM Na acetate, 40mM K acetate, 10mM glucose, 100mM sucrose, 1.2mM $MgCl_2$, 1mM $CaCl_2$, 100mM HEPES, pH 7.4) and fixed for 10 minutes at room temperature in 2X oocyte fix buffer (8% formaldehyde in 200mM cacodylic acid, 200mM sucrose, 80mM potassium acetate, 20mM sodium acetate, 20mM EGTA, pH 7.2) (Theurkauf and Hawley, 1992). Ovaries were rinsed in 0.1% PBT and transferred in a drop to a microscope slide, where stage 14 oocytes were individually hand dechorionated using dissecting needles (Precision Glide- size 27G 11/4) to allow penetration of antibodies. Stage 14 oocytes were transferred to microcentrifuge tubes, extracted in 1% PBT for 2 hours at room temperature, and stained overnight at 4^oC with anti-a-tubulin-FITC conjugated antibody (DM1A clone, Sigma) at 1:200 dilution in 0.1% PBT. Samples were washed in PBT, incubated with 800 mg/ml RNAse A (Qiagen) in PBS for 1 hour at 37^oC, washed again in PBT, incubated with 10 mg/ml propidium iodide in PBT for 15 minutes at room temperature, and washed in PBT. For visualization of DNA and spindles during embryonic mitoses, 0-3 hour embryos were collected on molasses agar plates, dechorionated in 3% NaOCl in water for 2 minutes, rinsed extensively with double-

distilled H_20 and transferred into a scintillation vial containing 5 ml of heptane (Sigma). An equal volume of methanol was then added and embryos shaken vigorously for 2 minutes. Samples were rinsed and washed three times for 5 minutes in methanol, fixed overnight at 4°C in 1 ml of methanol, blocked in PBT plus 5% normal goat serum (Jackson Immuno Research Labs) and 5% bovine serum albumin (Sigma) for 1 hour at room temperature, and stained with anti-a-tubulin-FITC conjugated antibody as above. All samples were mounted in Vectashield and analyzed using a Zeiss LSM 510 confocal microscope.

For X-gal staining, ovaries were dissected and teased apart in Grace's insect medium and fixed for 8 minutes at room temperature with 0.5% glutaraldehyde in Grace's. Ovaries were washed extensively in PBT and stained at 37°C for 20-30 minutes in 10 mM NaH_2PO_4-Na_2HPO_4 (pH 7.2), 150 mM NaCl, 1 mM $MgCl_2.6H_2O$, 3 mM K4[FeII$(CN)_6$], 3 mM K_3[FeIII$(CN)_6$], 0.5% Triton X-100, 0.2% X-gal. Staining reaction was stopped by washing ovaries in PBT for 30 minutes. Samples were mounted on 50% glycerol in PBS and analyzed using a Zeiss Axioplan 2 microscope.

Statistical analysis

For statistical analysis, results were subjected to chi-square test using the *Yates correction* ($\chi^2 = \Sigma(|O-E| - ½)^2 / E$).

Live imaging

Two to three day-old females fed with wet yeast paste were dissected in halocarbon oil 700 (Sigma) on a coverslip. Stage 13 egg chambers were placed on the coverslip with the dorsal side facing down and injected with a mixture of 1:20 OliGreen dye (Invitrogen) and 2 mg/ml rhodamine-labeled tubulin (Cytoskeleton, Inc.) in water. Live images were obtained at 20 second intervals using a Leica TCS SP5 inverted confocal microscope, and assembled into movies using Image J 1.39. Nuclear envelope breakdown duration was measured as the elapsed time from beginning of nuclear envelope ruffling until entry of tubulin into nucleus.

***Drosophila in vitro* expression cloning (DIVEC) binding screen**

To perform the DIVEC binding screen, I used the first release of the *Drosophila* Gene Collection (DGC), which consists of sequence-verified cDNAs encoded by 5,856 unique genes in the *Drosophila* genome, as described (Lee et al., 2005). Briefly, individual cDNA clones were grown in a 96-well format and pooled for DNA isolation. Each pool (containing 24 cDNAs) was *in vitro* transcribed/translated using the Promega TNT system and converted into pools of [^{35}S]-labeled proteins. I performed a primary screen by incubating 10 ml settled glutathione-sepharose beads bound to recombinant glutathione S-transferase (GST)-Dendos fusion protein with 1.5 ml of each radiolabeled 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 tables, EDTA free [Roche]). Reactions were incubated at 4°C in a micro tube mixer (Tomy Seiko) for 2 hours and then transferred to Wizard minicolumns (Promega) attached to a vacuum source. Minicolumns

were washed 3 times with 2.5 ml of Buffer A and once with 2.5 ml of Buffer B (50mM Tris, pH 8.0, 50 mM NaCl, 1 mM PMSF). After the final wash, minicolumns were transferred to microcentrifuge tubes and spun for 1 minute at 10,000 rpm in a tabletop centrifuge to remove excess wash buffer. Bound proteins were eluted in 95°C pre-heated 2X sample buffer, electrophoresed and detected by PAGE autoradiography. I found 5 pools with potential Dendos-interacting proteins. I then performed secondary and tertiary screens with four cDNAs per subpool or individual cDNAs, respectively, using GST beads as negative control and following the steps above. Phosphoimager analysis was used to determine the percentage of input protein bound to Dendos beads.

Mouse tissue analysis

All mice in the present investigation were housed and used in accordance with the National Institutes of Health and institutional guidelines on the care and use of laboratory animals.

For RT-PCR of mouse ovaries, estrous cycles of virgin CD-1 females (Charles River Laboratory) were classified according to morphology from vaginal smears. Females were mated with fertile CD-1 males to induce pregnancy, and ovaries were collected on day 5 of pregnancy (day 1 = vaginal plug).

Immunohistochemistry of 5 mm-thick Bouin's-fixed paraffin-embedded ovarian sections was achieved as previously described (Tan et al., 1999). In brief, after deparaffinization and hydration, sections were subjected to antigen retrieval using a pressure cooker in 10 mM sodium citrate solution (pH 6.0) for 20 minutes, and stained

with rabbit-anti Dendos c302 antibody at 1:1,000 dilution using the Histostain-Plus kit (Zymed).

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* (Chapter IV). 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 asked whether meiosis was affected in $endos^{00003}$ females by visualizing the oocyte DNA morphology with 4',6-diamidino-2-phenylindole (DAPI) (Figure 2.1, Table 2.1). As previously described (King, 1970), wild-type oocytes are in prophase I until stage 12 (Figure 2.1B-F,V). In early stage 13, only 5.6% of oocytes have progressed to metaphase I, while in mid stage 13, that percentage increases to 21%; by late stage 13, virtually all oocytes are in metaphase I, and this arrest is maintained in mature stage 14 oocytes. *endos* mutant oocytes arrest in prophase I, as indicated by the typical nuclear morphology; however, this arrest lasts longer, with 90% of the mid stage 13 and 58% of late stage 13 oocytes still in prophase I (Figure 2.1G-K,V). By stage 14, $endos^{00003}$ oocytes have exited prophase I, but only 3% are in metaphase I. Instead of progressing into metaphase I, 93% of the oocytes display dispersed or visually undetectable DNA. Heteroallelic $endos^{00003}/endos^{EY1105}$ and hemizygous $endos^{00003}/Df(3L)ED4536$ females show similar phenotypes (Table 2.1). Endos protein is normally expressed throughout the cytoplasm of ovarian germ cells

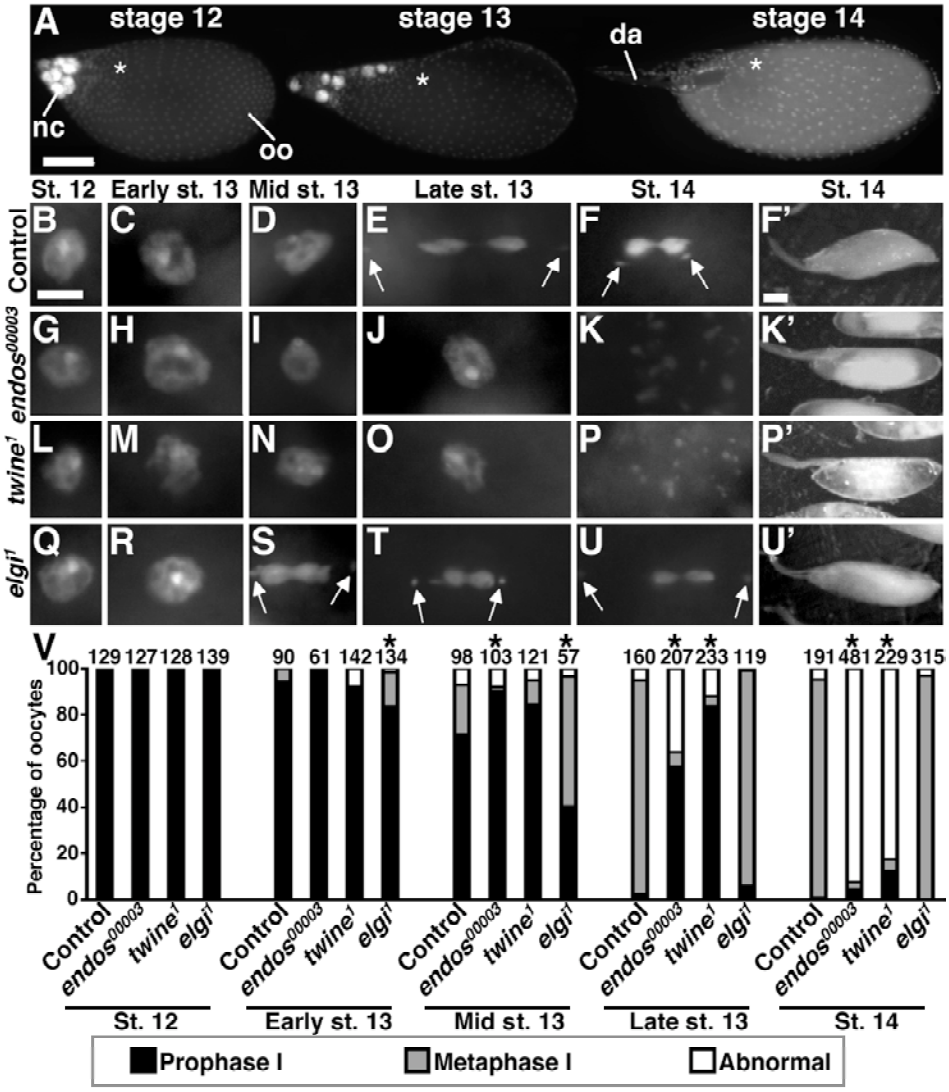

Figure 2.1. *endos*^{00003} oocytes fail to undergo meiotic maturation. (A) DAPI-stained egg chambers in different stages. nc, nurse cells; oo, oocyte; da, dorsal appendages. Asterisks indicate position of oocyte nucleus. Scale bar, 100 μm. (B-F) Control oocytes in prophase I (B-D) and in metaphase I (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. (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 non-dehydrated and have abnormal yolk. Scale bar, 100 μm. (V) Quantification of DNA morphology. Number of oocytes analyzed shown above bars. Asterisks, $P<0.001$.

Table 2.1. Quantification of oocytes arrested in prophase and metaphase of meiosis I

Strain[a]	Percentage of oocytes														
	Stages 10-12			Early stage13[b]			Mid stage 13[b]			Late stage 13[b]			Stage 14		
	PI[c]	MI[c]	Ab[c]	PI	MI	Ab	PI	MI	Ab	PI	MI	Ab	PI	MI	Ab
Control[d]	100	0 (129)[e]	0	94	6 (90)	0	71	21 (98)	7.1	2.5	93 (160)	5	1	94 (191)	4.7
***endos*^{00003}**	100	0 (127)	0	100	0 (61)	0	90	1.9 (103)	7.8	58	6.3 (207)	36	4.6	2.9 (481)	93
***endos*^{00003}/ *endos*^{EY1105}**	100	0 (50)	0	100	0 (37)	0	98	0 (40)	2.5	76	3.2 (63)	21	6.7	0 (60)	93
endos*^{00003}/ *Df(3L)ED4536	99	0 (89)	1.1	90	0 (29)	10	96	0 (25)	4	33	1.6 (64)	66	0.8	0 (124)	99
***twe*^{1}**	100	0 (128)	0	92	0.7 (142)	7.7	84	11 (121)	5	84	4.3 (233)	12	12	5.2 (229)	83
twe*^{1}*/Df(2L)RA5	32	0 (37)	68	46	2.3 (44)	52	46	4.4 (68)	50	42	11 (65)	48	13	7.3 (150)	80
***elgi*^{EY10782}** (Control)	100	0 (79)	0	93	6 (84)	1	60	40 (35)	0	1	98 (143)	1	0	98 (104)	2
***elgi*^{1}**	100	0 (139)	0	84	15 (134)	1.5	40	56 (57)	3.5	5.9	93 (119)	0.8	0	97 (315)	3.1
***elgi*^{1}/ *elgi*^{2}**	100	0 (28)	0	65	34 (114)	0.9	13	87 (54)	0	0.9	99 (116)	0	0	98 (92)	2.2
elgi*^{1}/ *Df(3L)brm11	100	0 (53)	0	83	17 (30)	0	35	65 (40)	0	0.9	99 (108)	0	0	100 (186)	0.5
***endos*^{00003}; *elgi*^{1}**	100	0 (105)	0	97	0 (31)	3.2	96	0 (24)	4.2	49	1.6 (61)	49	3.3	0.8 (121)	96
Control (29°C)[d]		n.d.		44	51 (41)	5	21	73 (66)	6	1	86 (80)	13	0	92 (12)	8
***cdc2*$^{E1-24}$/*cdc2*^{B47}** (29°C)	100	0 (100)	0	100	0 (169)	0	92	3 (105)	5	49	32 (250)	19	4	18 (626)	78

[a]Experiments were performed at 22-25°C, except were indicated (29°C). [b]Stage 13 oocytes were categorized as early, mid and late according to the number of nurse cell nuclei present: Early stage 13 egg chambers have 11-13 nurse cell nuclei, mid stage 13 egg chambers have 6-10 nurse cell nuclei, and late stage 13 egg chambers have 1-5 nurse cell nuclei. [c]PI, prophase I arrest; MI, metaphase I arrest; Ab, abnormal DNA morphology. [d]*yw* females were used as the wild-type control. [e]The number of oocytes analyzed is shown in parentheses.

(Drummond-Barbosa and Spradling, 2004), including stage 14 oocytes (J.R.V.S. and D.D.-B., unpublished). Moreover, germline-specific expression of Endos rescues meiotic maturation and fertility in *endos*^{00003} females (see Figure 2.12C,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 oocyte show metaphase I arrest, with 84% still in prophase I (Figure 2.1L-P,V). At stage 14, 83% of *twine*^{1} oocytes show abnormalities similar to those of *endos* mutants. Hemizygous *twine*^{1}*/Df(2L)RA5* females have similar defects (Table 2.1). In addition, using a temperature-sensitive *cdc2* mutant genotype, we also find direct evidence that Cdk1 activity is required for meiotic maturation in *Drosophila*. At the restrictive temperature (29^{o}C), *cdc2*$^{E1-24}$*/cdc2*^{B47} oocytes show prolonged prophase I at stage 13 and abnormal DNA morphology at stage 14 (Figure 2.2A-L, Table 2.1). Unfortunately, we could not examine the role of Cyclin B in meiotic maturation because it is required earlier role in oogenesis (Lin et al., 2005). The similarity between the *endos*^{00003}, *twine*^{1} , and *cdc2*$^{E1-24}$*/cdc2*^{B47} meiotic defects suggests that *endos* may regulate the progression from prophase I to metaphase I via the regulation of *twine* to control Cdk1 activity.

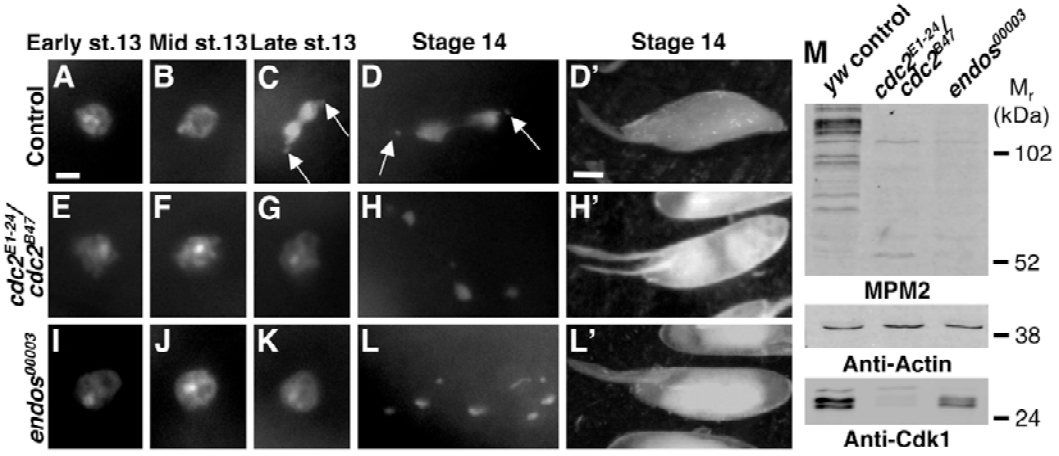

Figure 2.2. Cdk1 is required for meiotic maturation and for generation of MPM2 phosphoepitopes in *Drosophila*. (A-D) DAPI-stained control oocytes in prophase I (A,B) or metaphase I (C,D). Arrows indicate non-exchange fourth chromosomes. (E-H) Oocytes from $cdc2^{E1\text{-}24}/cdc2^{B47}$ females at the restrictive temperature have a prolonged prophase I (E-G) and abnormal DNA morphology at stage 14 (H) (see Table 1 in supplementary material). These phenotypes are similar to those observed in $endos^{00003}$ oocytes (I-L). Scale bar for A-L, 5 μm. (D') Control dehydrated oocyte. *cdc2* mutant oocytes (H') fail to dehydrate, similar to *endos* mutant oocytes (L'). Scale bar for D',H',L', 100 μm. (M) Western blotting using MPM2 antibodies, which recognize conserved phosphoepitopes generated by Cdk1 and Polo kinase in many species, and anti-Cdk1 (anti-PSTAIR) antibodies. Wild-type oocytes show high MPM2 levels, while both $endos^{00003}$ and $cdc2^{E1\text{-}24}/cdc2^{B47}$ have drastically reduced MPM2 levels. Thirty stage 14 oocytes per lane. Actin used as a loading control.

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

We next asked 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 (Figure 2.3). As expected, wild-type oocytes had intact nuclear envelopes in prophase I, while 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 (Figure 2.4). In wild-type oocytes, the nuclear envelope disassembled in ~9 minutes (n=3; measured times were 16, 4, and 6 minutes). In 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-a-tubulin-fluorescein isothiocyanate (FITC)-conjugated antibodies and propidium iodide to visualize microtubules and DNA,

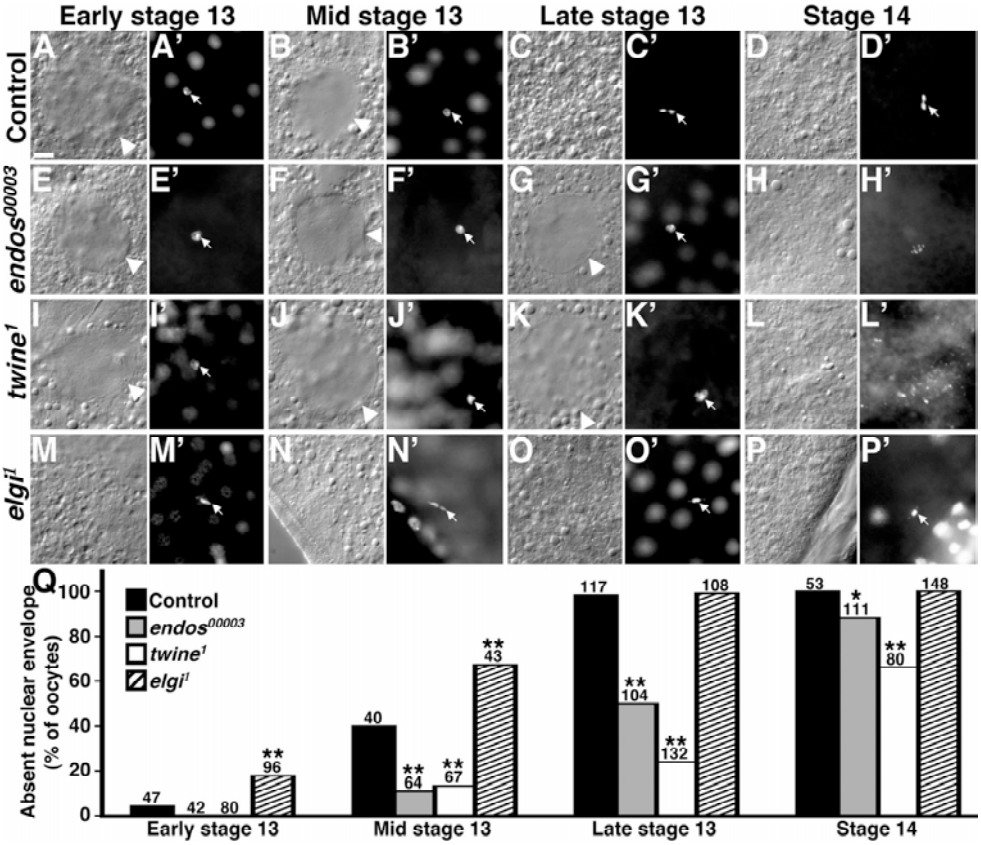

Figure 2.3. Nuclear envelope breakdown is delayed in *endos*^{00003} mutants. (A-P) Nomarski images of oocytes showing absent or present nuclear envelopes. (A-D) *yw* control oocytes showing intact nuclear envelope (A,B) during prophase I or absent nuclear envelope (C,D) after meiotic maturation. (E-H) *endos*^{00003} oocytes showing intact nuclear envelope until late stage 13 (E-G) but absent at stage 14 (H). (I-L) Delayed nuclear envelope breakdown of *twine*^{1}oocytes. (M-P) Premature nuclear envelope breakdown of *elgi*^{1} oocytes. Arrowheads indicate nuclear envelope. (A'-P') DAPI images for oocytes shown in A-P, respectively. Arrows indicate oocyte DNA. Scale bar, 10 mm. (Q) Percentage of oocytes showing absent nuclear envelope at different stages. Numbers above bars represent the total number of oocytes analyzed. Single asterisk, $P<0.01$; double asterisks, $P<0.001$.

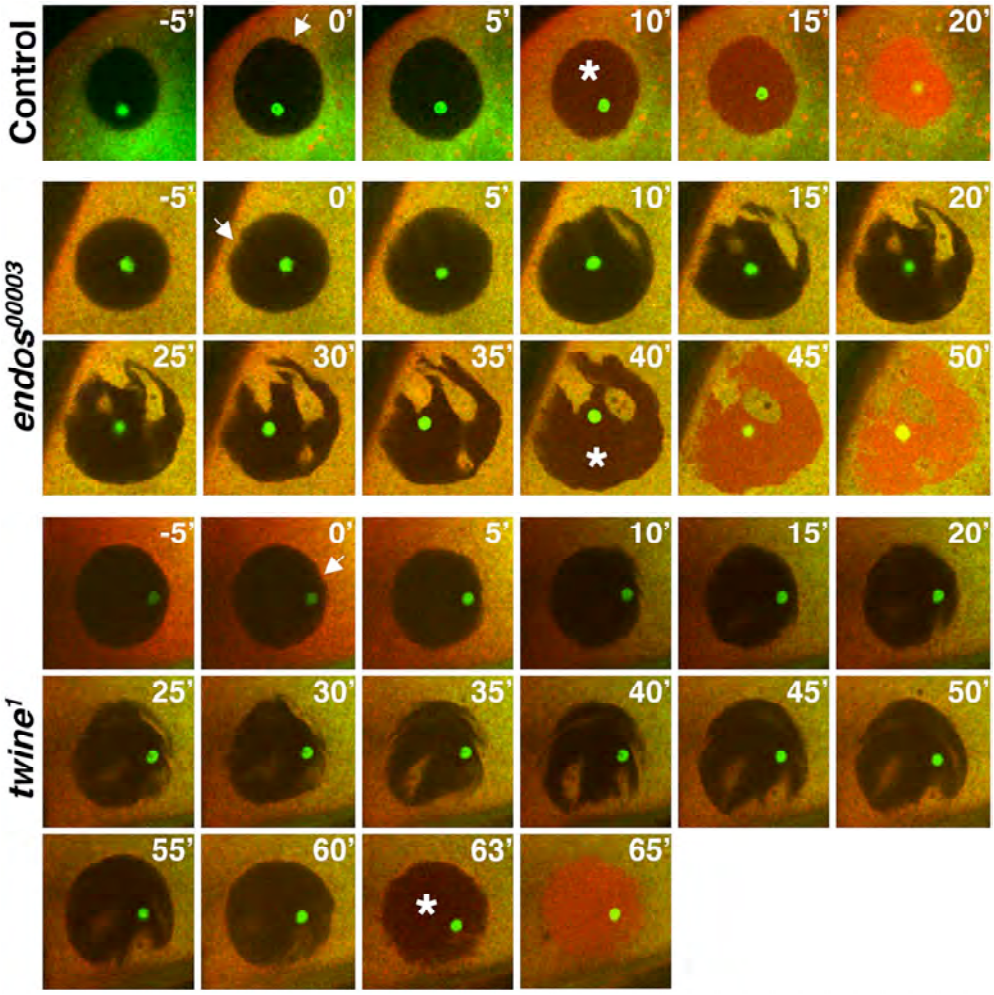

Figure 2.4. Live imaging of nuclear envelope breakdown in *endos*^{00003} and *twine*^{1} mutants. Control, *endos*^{00003}, and *twine*^{1} stage 13 oocytes injected with Oligreen to label DNA (green) and rhodamine-labeled tubulin (red) were analyzed using live imaging. Still images corresponding to time points at every 5 minutes are shown, arbitrarily starting 5 minutes prior (-5') to initiation of nuclear envelope breakdown (0', arrows). Nuclear envelope breakdown duration was measured as the elapsed time from beginning of nuclear envelope ruffling (arrows) until entry of rhodamine-labeled tubulin into nucleus (asterisks).

respectively (Figure 2.5A-F). While 92% (n=25) of control mature oocytes have the typical metaphase I elongated bipolar spindle (Figure 2.5A), this is rarely the case in *endos* or *twine* mutants. Instead, 87% (n=31) of *endos*^{00003} mutants fail to form or maintain the meiotic spindle at stage 14 (Figure 2.5B) and a small fraction (13%, n=31) have abnormal spindle-like structures attached to the dispersed DNA (Figure 2.5C). Most of *twine* mutant stage 14 oocytes (82%, n=22) also do not have a meiotic spindle (Figure 2.5D); only 7.7% show normal spindle formation, whereas 18% show abnormal spindle masses with DNA attached (Figure 2.5E). Live imaging indicated that the spindle either fails to form or fails 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 may have a similar role during early embryonic mitoses. We therefore looked at spindle formation in 0-3 hour embryos derived from *endos*^{00003} and *twine*^{1} females (Figure 2.5G-O). The majority of wild-type embryos (95%, *n*=95) showed normal mitotic spindles (Figure 2.5G). In contrast, 98% (*n*=51) of *endos*^{00003}-derived embryos had dispersed (Figure 2.5H) or undetectable DNA, resembling the stage 14 oocyte defect. Of those, about 25% had abnormal spindles associated with DNA masses (Figure 2.5I). Approximately 2% of *endos*^{00003}-derived embryos appeared to initiate mitotic divisions, but displayed abnormal bipolar, tripolar or multipolar spindles (Figure 2.5J). In accordance with previously reports (White-Cooper et al., 1993), the majority of *twine*^{1}-derived embryos (96%, *n*=74)

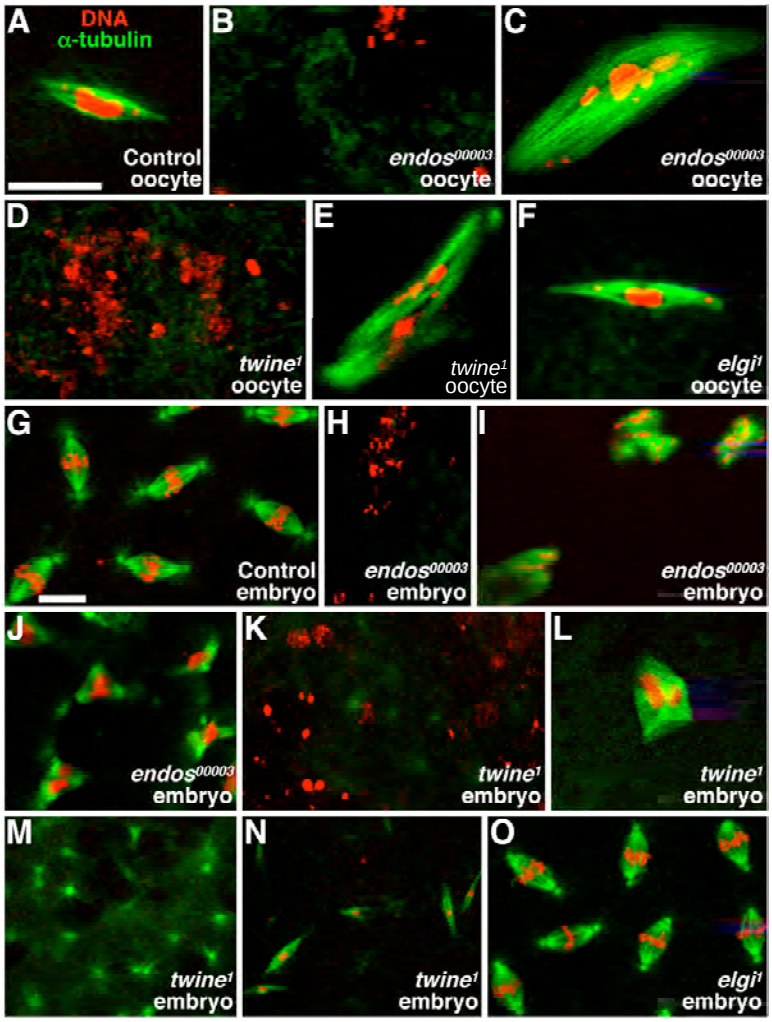

Figure 2.5. *endos*^{00003} mutants have spindle defects. Stage 14 oocytes (A-F) and 0-3 hour embryos (G-O) stained with propidium iodide (DNA, red) and anti-α-tubulin (microtubules, green). (A) Control oocytes in metaphase I. (B) Typical *endos*^{00003} oocyte showing dispersed DNA without a spindle. (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.

also showed dispersed (Figure 2.5K) or undetectable DNA. Half of those had abnormal spindles associated with DNA masses (Figure 2.5L), while 8% had free spindle asters (Figure 2.5M) and/or thin long spindles (Figure 2.5N). 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 at stage 14 (Figure 2.1F',K',P'; Figure 2.2H',L'). To address whether *endos* may regulate *twine*, we examined the levels of a functional Twine::β-galactosidase (Twine::b-gal) fusion protein (Maines and Wasserman, 1999; White-Cooper et al., 1998) in *endos* mutants. In control and *endos*^{00003} ovarioles, Twine::b-gal expression is low or undetectable until stage 12, and detectable at stage 13. At stage 14, however, Twine::b-gal expression is stronger in control but greatly reduced in *endos*^{00003} oocytes (Figure 2.6A,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. In contrast, expression of Myc::Twine was severely reduced in *endos*^{00003} females, and these low Myc::Twine levels did not rescue

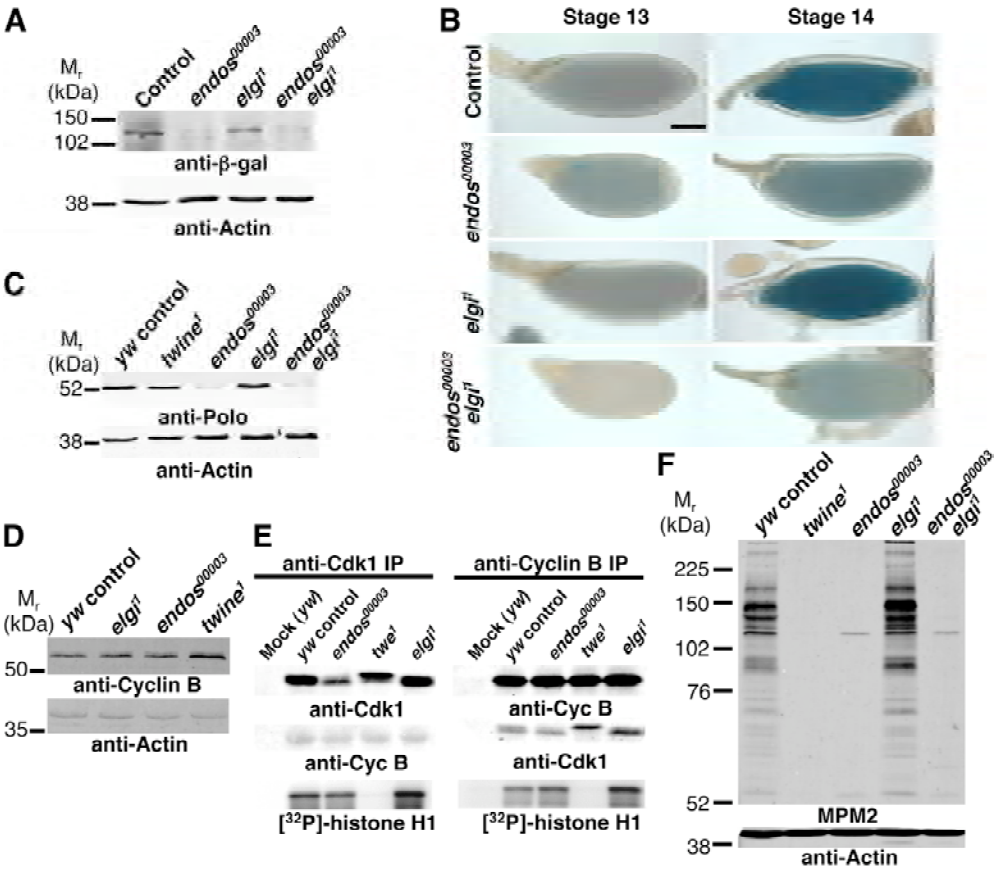

Figure 2.6. Endos regulates Twine, Polo, and MPM2 phosphoepitopes. (A) Anti-b-gal western blotting showing reduced expression of Twine::β-gal in *endos*^{00003} and *endos*^{00003} *elgi*^{1} stage 14 oocytes. Thirty stage 14 oocytes per lane. (B) X-gal staining (blue) of control, *endos*^{00003}, *elgi*^{1}, and *endos*^{00003} *elgi*^{1} oocytes reflecting Twine::β-gal levels at stages 13 and 14. Scale bar, 100 mm. (C) Polo western analysis of control, *twine*^{1}, *endos*^{00003}, *elgi*^{1}, and *endos*^{00003} *elgi*^{1} stage 14 oocytes showing strong reduction of Polo in *endos*^{00003} and *endos*^{00003} *elgi*^{1}. Fifty stage 14 oocytes per lane. (D) CyclinB western analysis showing normal expression in *endos*^{00003} and *elgi*^{1} mutants or slightly elevated in *twine*^{1} mutants. Thirty stage 14 oocytes per lane. (E) In vitro Cdk1 kinase assay using anti-Cdk1 or anti-CyclinB 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-CyclinB 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, while *endos*^{00003}, *twine*^{1} and *endos*^{00003} *elgi*^{1} oocytes have drastically reduced MPM2 phosphoepitopes. Fifty stage 14 oocytes per lane. Actin used as a control.

the *endos*^{00003} defects (Figure 2.7A,B; 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 Cdk1/Cyclin B activation (Kumagai and Dunphy, 1996; Qian et al., 2001). In mice, Cdk1/Cyclin B-mediated phosphorylation stabilizes Cdc25A and Cdc25B, creating a positive feedback loop (Mailand et al., 2002; Nilsson and Hoffmann, 2000). We therefore asked 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 (Figure 2.8), although *polo* mRNA expression was unaffected in *endos*^{00003} oocytes (D.D.-B. and J.R.V.S., unpublished), indicating a posttranscriptional effect. The reduced levels of Polo kinase and Twine in *endos* mutants are not due to a generalized effect on protein expression, as they show no decrease in Cyclin B (Figure 2.6D), 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 Figure 2.7C,D). Thus, we could not determine if Polo expression is sufficient to rescue the *endos* defects.

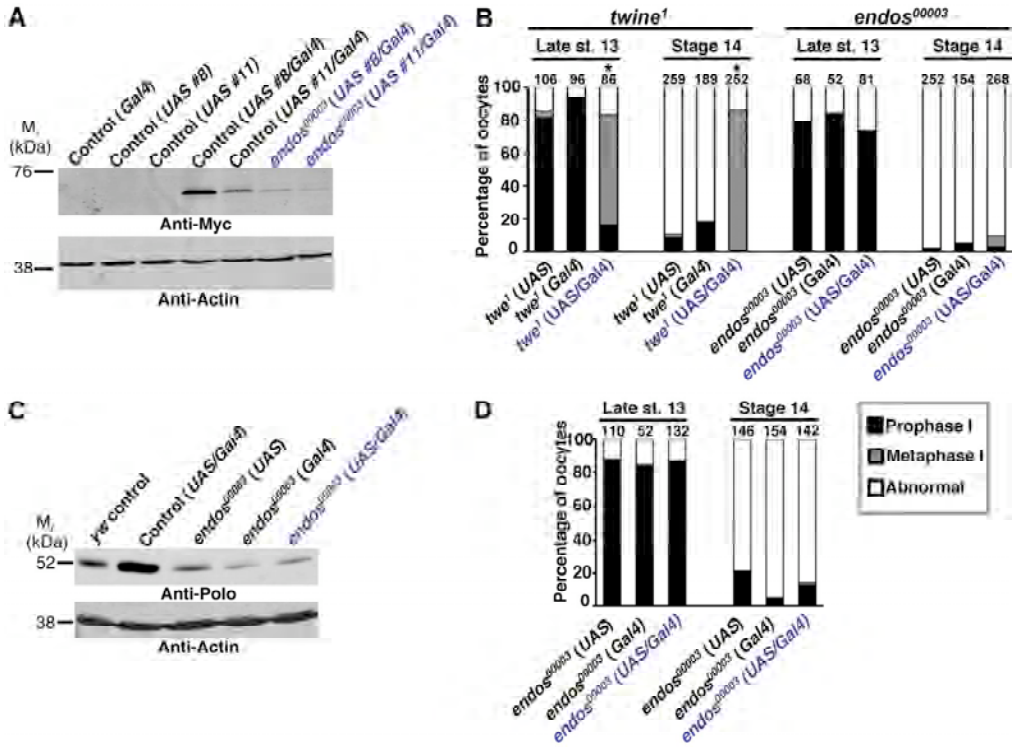

Figure 2.7. Meiotic defects of *endos* mutants cannot be rescued by Twine or Polo expression. (A) Western blot of control and *endos*^{00003} ovaries expressing *myc::twine* fusion transgenes. The germline-specific *nanos-Gal4::VP16* driver drives robust Myc::Twe expression from *UAS-myc::twine #8* or *#11* transgenes in control ovaries, but reduced levels are detected in *endos*^{00003} mutants. (B) Rescue of meiotic defects and sterility of *twine*^{1} mutants by *nanos-Gal4::VP16*-induced expression of the *UAS-myc::twine #8* transgene but failure of this functional transgene to rescue the *endos*^{00003} mutant defects. (C) Western blot of control and *endos*^{00003} mutant ovaries expressing a functional *UASp-polo* transgene (Xiang et al. 2007). Robust Polo expression induced by the germline-specific *nanos-Gal4::VP16* driver in control females, but reduced expression levels induced in *endos*^{00003} mutants. One pair of ovaries (enriched for stage 14 oocytes by starving females) per lane. Actin used as a loading control in A,C. (D) Failure of *nanos-Gal4::VP16*-induced expression of the *UASp-polo* transgene to rescue the meiotic defects of *endos*^{00003} mutants. In B,D, numbers above each bar, number of oocytes analyzed; asterisks, $P<0.001$; control genotypes shown in black, and experimental genotypes shown in blue.

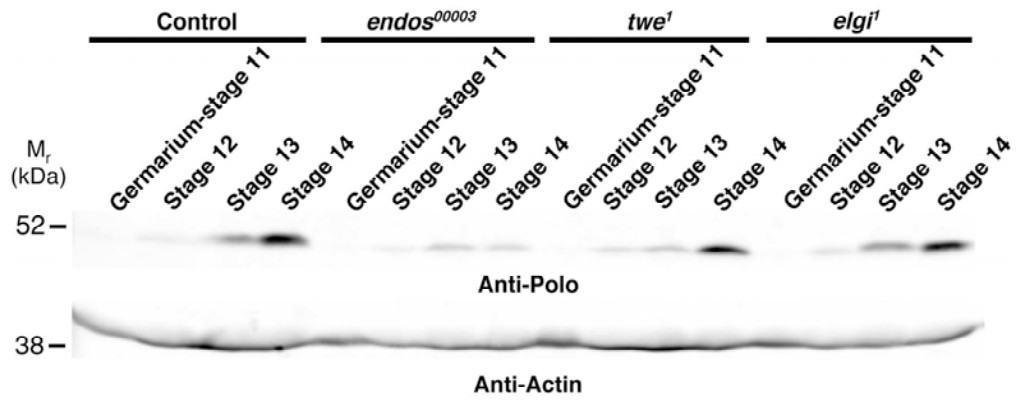

Figure 2.8. Polo kinase levels are markedly reduced in *endos*^{00003} mutants. Polo western analysis of egg chambers at different developmental stages. Wild-type and *elgi*^{1} ovarioles show low Polo expression from germarium to stage 12 and increased Polo expression at stages 13 and 14. *twine*^{1} stage 13 and 14 oocytes show slight reduction in Polo levels, while *endos*^{00003} oocytes show stronger reduction. One-hundred egg chambers or oocytes per lane. Actin used as a loading control.

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

The low Polo and Twine levels may 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) (Figure 2.6E). Similar results were obtained with anti-Cyclin B immunoprecipitates, suggesting that stage 14 oocytes contain active Cdk1/Cyclin B complexes. *twine*^{1} immunoprecipitates had markedly reduced kinase activity (R.I.=0.13$\pm$0.11; n=8; p<0.001). In contrast, Cdk1 in *endos*^{00003} and *elgi*^{1} immunoprecipitates had normal kinase activity (1.52$\pm$0.96, n=9, and 1.47$\pm$1.02, n=8, respectively; p>0.05 for both), although Cdk1 was present at slightly reduced levels and had altered electrophoretic mobility (appeared hypophosphorylated) in *endos*^{00003} mutants (Figure 2.6E; Figure 2.2M). 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 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, while in *cdc2*$^{E1\text{-}24}$/*cdc2*^{B47} mutants they are severely reduced, indicating that 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 Cdk1 activity *in vivo* (Figure 2.6F; Fig. 2.2M).

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

To identify proteins that directly bind to Endos and better understand its role in the meiosis, we performed a *Drosophila in vitro* expression cloning binding screen (Figure 2.9) modified from the approach previously used to screen for kinase substrates (Lee et al., 2005). Sequence-verified cDNAs corresponding to 5,856 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 (Figure 2.9B).

Elgi has a highly conserved RING finger domain and it has vertebrate homologs (Figure 2.9C), including 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, sharing 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 (Figure 2.9B), but were unable to generate high quality anti-Elgi polyclonal antibodies to confirm the Endos-Elgi interaction *in vivo*. We detected *elgi* and *CG9014*

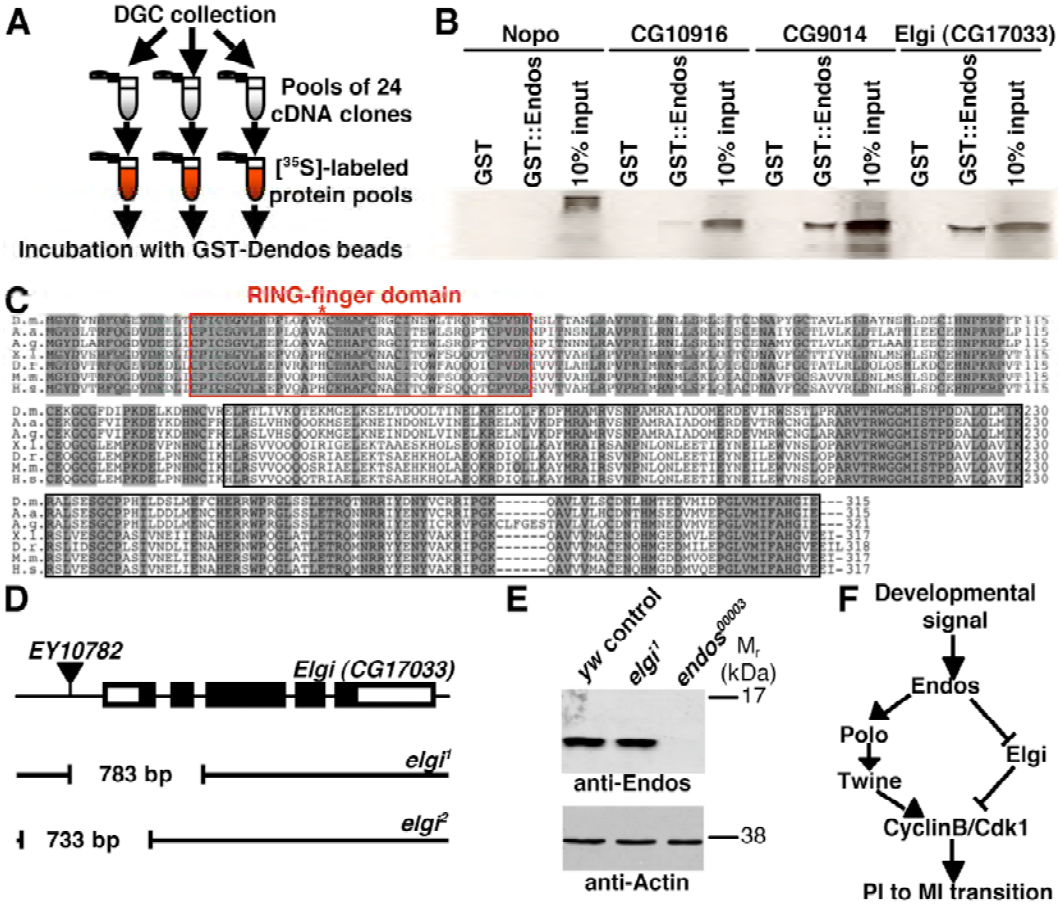

Figure 2.9. Endos binds to a putative E3 ubiquitin ligase encoded by *elgi*. (A) DIVEC screen used to identify Endos interactors. (B) Autoradiogram showing that the GST::Endos fusion protein specifically binds to the closely related CG9014 and Elgi E3 ubiquitin ligases but not to more distant E3s, such 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. Shaded amino acid residues represent identities. Asterisk indicates the first methionine for 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 showing normal Endos levels in *elgi^1* ovaries. Actin used as loading control. (F) Model for the role of Endos in oocyte meiotic maturation. Endos controls Polo kinase and, perhaps indirectly, Twine levels, leading to activation of CyclinB/Cdk1. By binding and inhibiting Elgi, Endos may have a parallel role in refining the timing of meiotic maturation.

mRNA expression in heads and carcasses but *elgi* predominates in ovaries (Figure 2.10A).

elgi mutation results in premature metaphase I

To examine the role of *elgi* in meiotic maturation, we generated two deletion alleles (Figure 2.9D). $elgi^{1}$ is likely a null allele because no mRNA is detected in homozygotes (Figure 2.10B). Disruption of *elgi* results in semi-lethality, indicating a role during development. $elgi^{2}$ lacks a small portion of the *elgi* coding region (Figure 2.9D), and some mRNA is still detected in trans to $elgi^{1}$ (Figure 2.10B). Although $elgi^{1}$ females have normal ovarian morphology and are fertile, progression from prophase I to metaphase I occurs prematurely (Figure 2.1Q-V; Table 2.1), prompting the name *early girl*, or *elgi*. Nuclear envelope breakdown is also premature in $elgi^{1}$ mutants (Figure 2.3M-Q), with metaphase I spindles comparable to those of control oocytes (Figure 2.5F) and perhaps slightly elevated MPM2 levels (Figure 2.6F; clear increase in $elgi^{1}$ MPM2 levels observed in two out of three experiments). $elgi^{1}$/*Df(3L)brm11* hemizygotes show similar phenotypes (Table 2.1), 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

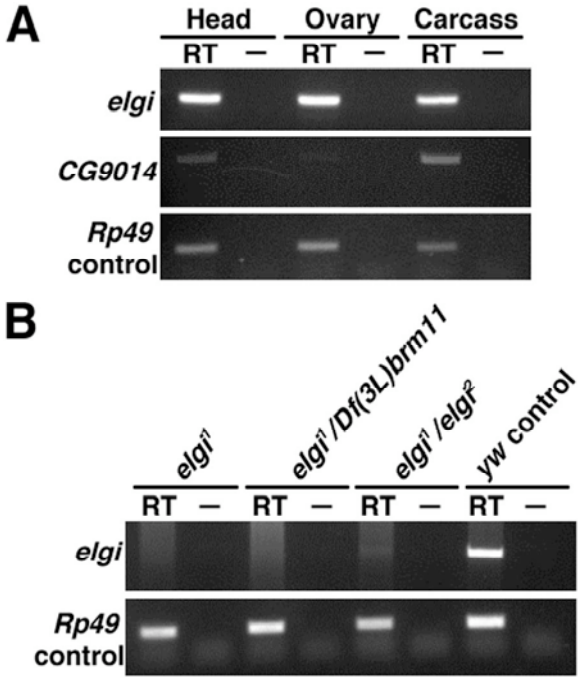

Figure 2.10. *elgi* mRNA is expressed in the ovary and other adult tissues. (A) RT-PCR analysis reveals that *elgi* and *CG9014* have distinct but overlapping expression patterns in adult tissues. Specifically, *elgi* is well expressed in the ovary, but the ovarian expression of *CG9014* is relatively weaker. (B) $elgi^{1}$ homozygous or hemizygous ovaries do not express any detectable *elgi* mRNA, suggesting that the $elgi^{1}$ allele is a molecular null. In contrast, ovaries from females carrying the $elgi^{2}$ allele express low levels of mutant *elgi* mRNA, predicted to encode a truncated protein lacking a functional RING finger domain (see Fig. 4D and Materials and Methods). Samples were normalized against *Rp49* as a control. RT reactions were performed in presence (RT) or absence (-) of reverse transcriptase.

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 do not observe any changes in Endos mobility or levels in *elgi*^{1} oocytes (Figure 2.9E). Endos may instead inhibit Elgi by blocking its interaction with target proteins. Because Polo kinase and Twine protein levels are reduced in *endos*^{00003} mutants, we asked 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 unaffected in *elgi*^{1} mutants (Figure 2.6A-C; Figure 2.8), suggesting that the 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 2.1). This is likely not due to redundancy between *elgi* and *CG9014* because loss of *elgi* function alone causes premature meiotic maturation (Figure 2.1V). Instead, we propose that Endos controls meiotic maturation via parallel mechanisms by modulating the protein levels of Polo kinase (and Twine) and also Elgi activity (Figure 2.9F).

The E3 RING family is defined by proteins that contain a zinc binding really interesting novel gene (RING) finger domain (Jackson et al., 2000). RING finger domains contain a series of conserved histidine and cysteine residues that serve to recruit the E2 enzyme to the substrate and as a cofactor to enhance substrate modification by the E2 (Jackson et al., 2000). To begin to investigate whether the Elgi E3 ligase activity is responsible for its meiotic functions, we simultaneously mutated the two conserved RING domain cysteines 54 (C54) and 63 (C63) into alanine to generate transgenic lines *elgi*^{C54,63A}*#7* and *elgi*^{C54,63A}*#12*. We hypothesized that mutations in the Elgi RING domain will impair its E3 ligase activity and will also result in premature entry into metaphase I.

Surprisingly, germline overexpression of Elgi^{C54,63A} by the *nanos::VP16-Gal4* driver resulted in a *endos*-like phenotype (Figure 2.11). As described before, only 2.5% of control late stage 13 oocytes remain in prophase I (93% of these oocytes have already entered metaphase I), whereas 58% of *endos*^{00003} oocytes are still in prophase I. Interestingly, 68% and 76% of oocytes overexpressing Elgi^{C54,63A}#7 and Elgi^{C54,63A}#12, respectively, show a dramatic prolonged prophase I. At stage 14, a very small fraction of control oocytes are found in prophase I (1%). However, the number of oocytes that are still in prophase I in *elgi*^{C54,63A}*#7* and *elgi*^{C54,63A}*#12* females , respectively, are 28% and 61%. These numbers are significantly higher than those observed in *endos*^{00003} mutants alone (5%). These results suggest that Elgi^{C54,63A} behaves as a dominant-negative protein that most likely does not have E3 ligase activity but that binds and sequesters endogenous Endos through its carboxy (C) terminus domain. Consistent with this prediction, we have indentified the Elgi C-terminus domain as the Endos binding domain *in vitro* (Min-Young Kim, unpublished). It will be important to determine in the near future the *in vivo* role of the Elgi C-terminus and whether mutations in this domain can affect the Elgi-Endos interaction and/or the role of *elgi* in oocyte meiotic maturation.

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 if *α-endosulfine* is also functionally conserved, we tested whether expression of ENSA, the human homolog, could rescue the *endos*^{00003} meiotic maturation defect (Figure 2.12 A-F). When *UAS-endos* transgenes were specifically expressed in the germline of *endos*^{00003} females, transition to metaphase I, stage 14

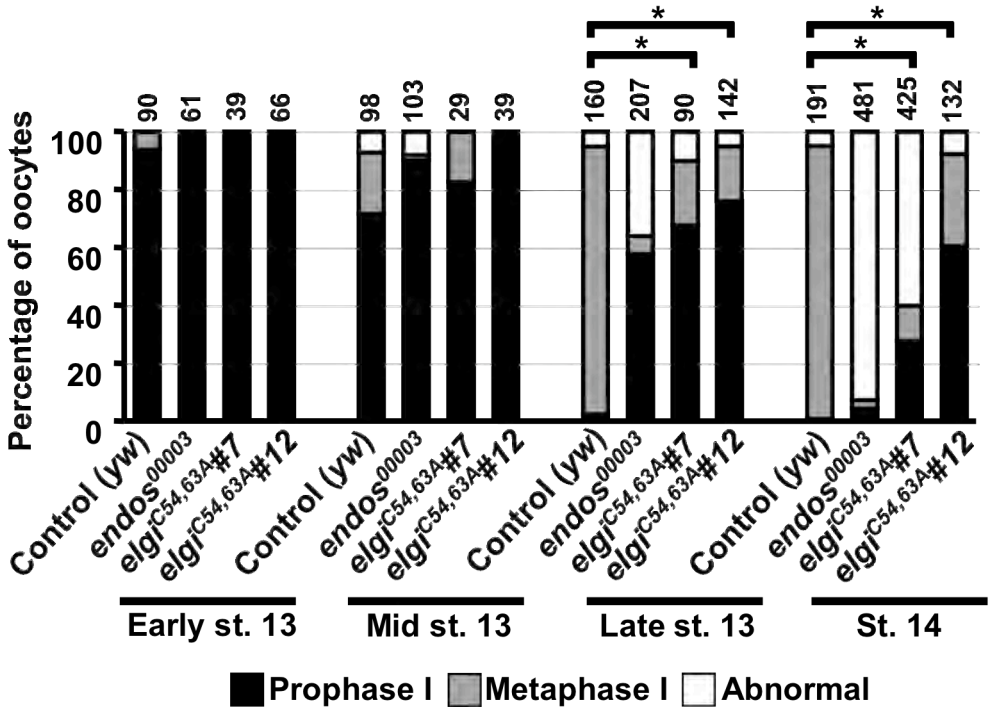

Figure 2.11. Mutations in Elgi RING domain cause an *endos*-like phenotype. Percentage of oocytes in prophase I and metaphase I or showing abnormal DNA morphology including fragmented or undetectable DNA. Transgenic lines *UASp-elgi^{C54,63A}#7* and *UASp-elgi^{C54,63A}#12* were crossed to the *nanos::VP16-Gal4* driver to induce overexpression of the Elgi C54,63A RING domain point mutations in the germline of wild-type flies. Flies overexpressing the mutated Elgi RING domain have a prolonged prophase I very similar to *endos^{00003}* mutants. Numbers above bars represent the total number of stage 13 or stage 14 oocytes analyzed. Asterisks, $P<0.001$. (Please note: This figure represents new data not included in the published manuscript.)

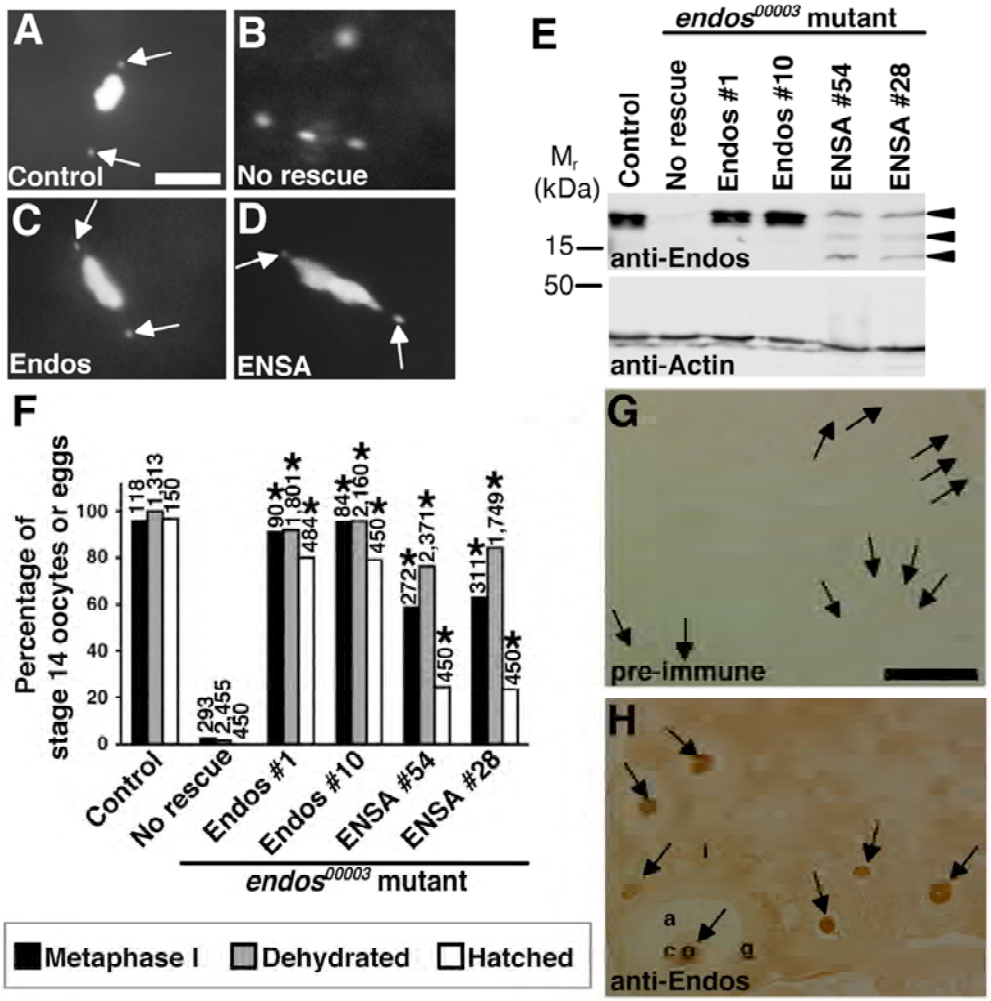

Figure 2.12. The function of α-endosulfine may be evolutionarily conserved. (A-D) DAPI-stained stage 14 oocytes from control and *endos*^{00003} females expressing Endos or human a-endosulfine (ENSA). Control oocytes (A) arrested in metaphase I and *endos* mutant oocytes (B) with dispersed DNA. *nanos-Gal4::VP16*-driven germline expression of *UAS-endos* (C) or *UAS-ENSA* (D) transgenes showing *endos* rescue. Arrows indicate fourth chromosomes. Scale bar, 5 mm. (E) Western analysis showing ovarian expression of Endos and ENSA in rescued females. Actin used as loading control. Arrowheads indicate ENSA-specific bands (lower bands are likely degradation products), absent in "No rescue" control. (F) Quantification of *endos*^{00003} rescue. Number of stage 14 oocytes (metaphase I arrest and dehydration) or eggs (hatch rate) analyzed shown above bars. Asterisks, $P<0.001$. (G,H) Mouse ovary immunohistochemistry showing that anti-Endos antibodies strongly label the cytoplasm of oocytes (H), while 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.

dehydration, and fertility were efficiently restored (Figure 2.12C,F). Remarkably, germline-driven *UAS-ENSA* transgenes also significantly rescued the *endos*^{00003} defects (Figure 2.12D,F), suggesting conservation of the molecular function of α-endosulfine.

We also asked if *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 Figure 2.12E), 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 (Figure 2.12G,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 loss of *endos* function. Due 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 indeed cause the *endos* meiotic maturation defects. In addition, our data suggest that Endos has a separate role during meiotic maturation via the negative regulation of Elgi. The function of α-endosulfine in meiotic maturation may potentially 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 and 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 Cdk1/Cyclin B results in mouse Cdc25A stabilization, thereby creating a positive feedback loop that allows Cdc25A to dephosphorylate and activate Cdk1/Cyclin B. Evidence from *Xenopus* studies indicates that phosphorylation and activation of Cdc25 by Polo-like kinase generate MPM2 epitopes, which reflect high Cdk1/Cyclin B 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 severe reduction in Polo and 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 hypophosphorylated) and *twine* (appears hyperphosphorylated, as expected) relative to 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 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, 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 directly confirm the Endos-Elgi interaction *in vivo*, our data indicating that the dominant-negative Elgi RING point mutant can still bind to Endos, producing an *endos*-like phenotype, are consistent with an *in vivo* interaction. Furthermore, Elgi was recently identified as an Endos *in vivo* interactor by mass spectrometry (David Glover, personal communication). Thus their strong interaction *in vivo* and *in vitro* interactions combined with the premature meiotic maturation phenotype of *elgi* mutants suggest that these genes function in the same pathway. The mammalian Elgi homologue, 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 modulation of Cdk1 has not been examined. The strong degree of amino acid similarity between human Nrdp1 and Elgi suggests 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 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; Endos may control the precise timing of meiotic maturation by inhibiting this E3 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 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 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 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 due to 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 (Chapter IV), differing from mammalian studies that link α-endosulfine to this process (Virsolvy et al., 2002; Virsolvy-Vergine et al., 1992). It is possible that this discrepancy is due to 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.

CHAPTER III

PROBING INTO THE MOLECULAR FUNCTION OF *ENDOS*

Introduction

As discussed in Chapters I and II, meiotic maturation triggers release from the prophase I arrest and progression into the metaphase I arrest. This process is characterized by key events including disassembly of the nuclear envelope, condensation of the chromosomes, and the formation of a bipolar spindle. In addition, meiotic maturation is tightly regulated in a temporal manner, and thus, disturbance of this regulation would not only impair the timing of meiotic maturation but could also affect the subsequent events.

The mechanisms underlying the temporal control of oocyte meiotic maturation remain to be fully elucidated in *Drosophila*. Recently, new mechanistic insights came from work by Xiang et al. (Xiang et al., 2007), which demonstrated that *Matrimony* (*Mtrm*) controls the timing of nuclear envelope breakdown in a dose-dependent manner. *Mtrm* was originally isolated from a deficiency screen for dominant effects on achiasmate chromosome segregation (Harris et al., 2003). According to the current model, prior to the onset of nuclear envelope breakdown, an excess amount of Mtrm inhibits Polo kinase activity via a stoichiometric physical interaction and, just before meiotic maturation, the destruction of Mtrm relieves this inhibition and frees Polo kinase to activate Twine, thereby leading to Cdk1/cyclin B-mediated nuclear envelope disassembly (Xiang et al., 2007). Consistent with this model and with the *Mtrm* dominant effect, females

heterozygous for a *Mtrm* null allele ($Mtrm^{\Delta 126}$) display precocious nuclear envelope breakdown (as early as in stage 11), a phenotype that can be significantly suppressed by the simultaneous removal of one copy of *polo* or *twine* (Xiang et al., 2007). Homozygosity of *Scott of the Antarctic* (*Scant*) mutation in the greatwall (*gwl*) gene, which results in hyperactivity of the conserved Greatwall kinase, reduces Polo levels and thus also suppresses the $Mtrm^{\Delta 126}$/+ phenotype (Archambault et al., 2007). (For a discussion of the potential role of *gwl* in *endos* pathway see Chapter V.) These data point to the regulation of Polo kinase activity as a key factor in determining the correct timing of meiotic maturation.

In addition to the defect in nuclear envelope breakdown timing, in $Mtrm^{\Delta 126}$*/TM3, Ser* females, the fourth achiasmate chromosomes fail to orient properly toward the poles, leading to a high frequency of achiasmate chromosomal non-disjunction that presumably accounts for the complete sterility of *Mtrm* homozygous females (Xiang et al., 2007). As discussed in Chapter I, *Drosophila* achiasmate chromosomes are held together by heterochromatic pairing rather than by the normal recombination events, and this "backup mechanism" is both necessary and sufficient to ensure the correct orientation of the fourth chromosomes centromeres, which leads to their correct position on opposite poles at metaphase I (see Figure 1.5B in Chapter I) and to their correct segregation (Hawley et al., 1992; Karpen et al., 1996). Interestingly, it has been shown that regulation of levels of Polo activity is also required during this process (Xiang et al., 2007). Prior to my studies, however, it was not clear whether or not the fourth chromosomal misorientation was simply a consequence of the premature nuclear envelope breakdown or an independent event.

My data presented in Chapter II indicate that, similar to *Mtrm* mutants, a fraction of *elgi* mutants undergo nuclear envelope breakdown prematurely. Unlike *Mtrm* or *elgi* mutants, however, I find that mutations in the *endos* gene induce a significant delay in the process of nuclear envelope breakdown, a phenotype that we also observe in *cdk1* and *twe* mutants. Taken together, these data and the recently published data suggest that interplay between these genes (*endos, Mtrm*, *elgi*, *polo*, *twe* and *gwl*) is required to set the right timing of nuclear envelope breakdown in the oocyte.

Two of our major goals in the laboratory are to understand the regulation of *endos* activity and to further elucidate how *endos* is regulating the downstream signaling pathways leading to nuclear envelope breakdown and progression into metaphase I. To begin pursuing these goals, I examined genetic interactions between *endos* and *Mtrm* and asked whether the conserved protein kinase A (PKA) phosphorylation site in α-Endosulfine (as discussed in Chapter I) is important for Endos' role in meiosis. In this chapter, I present the data obtained from these experiments, which show that *endos* and *Mtrm* genetically interact to regulate two distinct processes in meiosis (i.e. timing of nuclear envelope breakdown and orientation of achiasmate chromosomes to opposite poles). In addition, I provide data that show that Mtrm protein stability is affected in *endos* mutants and that suggest that dephosphorylation of Endos at stage 14 might be important to regulate the correct orientation of achiasmate chromosomes in metaphase I.

Materials and Methods

Control and experimental genotypes for phosphorylation site mutant experiments

Control genotypes were: (1) *yw*, (2) *endos*^{00003}, (3) *nanos-Gal4::VP16*, (4) *yw;UASp-endos*$^{S107A\#71}$*,endos*^{00003}*/ endos*^{00003}; (5) *yw;UASp-endos*$^{S107A\#75}$*,endos*^{00003}*/ endos*^{00003}, (6) *UASp-endos*$^{S107D\#13}$*/+;+;endos*^{00003}*/ endos*^{00003}. Experimental genotypes were: (1) *yw;UASp-endos*$^{S107A\#71}$*,endos*^{00003}*/ nanos-Gal4::VP16,endos*^{00003}, (2) *yw;UASp-endos*$^{S107A\#75}$*,endos*^{00003}*/ nanos-Gal4::VP16,endos*^{00003}, (3) *UASp-endos*$^{S107D\#13}$*/+;+;endos*^{00003}*/ nanos-Gal4::VP16,endos*^{00003}, (4) *yw;UASp-endos WT#1/+; endos*^{00003}*/ nanos-Gal4::VP16,endos*^{00003}.

Meiotic maturation and fourth chromosome orientation analysis

The analysis of meiotic maturation (based on the number of oocytes at prophase I or metaphase I) was performed as indicated in methods under Chapter II. The analysis of non-exchange fourth chromosome orientation on metaphase plate was performed by counting the number of oocytes having the fourth chromosomes, which can be identified based on their small size, at the same pole (misoriented).

Hatch rate measurements

Fertility was measured by performing hatch rate analysis. One-hundred and fifty eggs were collected from control *yw*, *Mtrm*$^{\Delta 126}$*/TM3, Ser or endos*^{00003}*/TM3, Sb*^{e} females and from *Mtrm*$^{\Delta 126}$ *+/+ endos*^{00003} transheterozygous experimental females and placed on molasses plates in groups of ten with a small amount of wet yeast at the center of the

plate (to keep larvae in the center). Plates were incubated in a humidity chamber at 25°C for two full days and the number of hatched eggs was counted. Experiments were performed in triplicate. Standard deviation was determined using the student *t*-test.

Western blot analysis and immunostaining

Western blot analysis was performed as described in Chapter II with minor modifications. To analyze Endos protein mobility, one-twentieth of one pair of ovaries was electrophoresed in a 17% polyacrylamide-SDS gel, transferred to a membrane and probed with anti-Endos antibodies. Immunostaining of embryos (0-20 minutes) was performed following same protocol as in Chapter II. The anti-Mtrm antibodies were provided by Dr. Scott Hawley (Stowers Institute).

Results

Precocious nuclear envelope breakdown in *Mtrm$^{\Delta 126}$*/+ heterozygotes is dominantly suppressed by *endos^{00003}*

Given the significant difference in the timing of nuclear envelope breakdown between *endos^{00003}* mutants and *Mtrm/TM3, Ser* heterozygotes, I hypothesized that these genes were antagonizing each other during meiotic maturation. To begin testing this hypothesis, I examined dominant genetic interactions by removing one copy of *endos* in a *Mtrm* heterozygous background (*Mtrm$^{\Delta 126}$* +/+ *endos^{00003}*) and analyzing meiotic maturation (Figure 3.1). Using the same criteria described in Chapter II, I analyzed DNA morphology using 4',6-diamidino-2-phenylindole (DAPI) staining at different egg chamber developmental stages, namely stage 11, early, mid and late stage 13, and stage

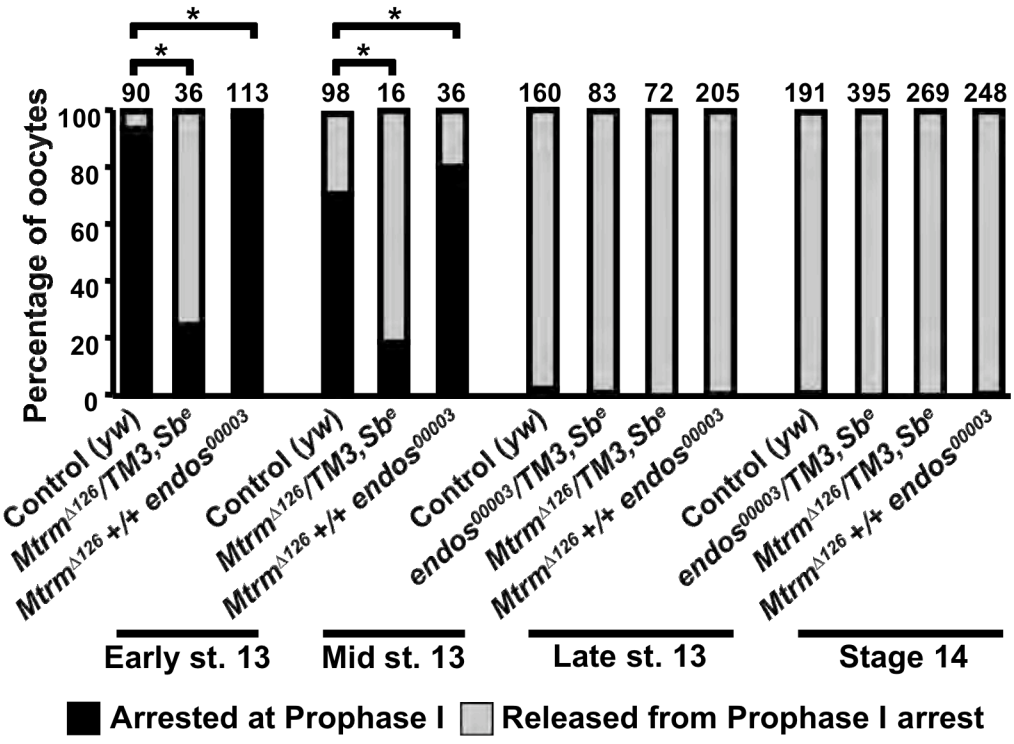

Figure 3.1. Reduction of *endos*^{00003} dosage strongly suppresses the premature meiotic maturation in *Mtrm*$^{\Delta 126}$*/TM3, Ser* heterozygotes. Percentage of oocytes arrested in prophase I or released from this arrest. Number above bars represent the total number of stage 13 and 14 oocytes analyzed. Asterisks, $P<0.001$.

14 oocytes. In wild-type control females, all oocytes at stage 11 were arrested in prophase I (*n*=50). Only 5.6% (*n*=90) of the early stage 13 oocytes were in metaphase I. By mid stage 13, 28.1% (*n*=98) of control oocytes have progressed to metaphase I. (Note: I have not quantified these early stages in *endos*^{00003}*/TM3, Sb*^{e} oocytes yet.) In contrast, in *Mtrm*$^{\Delta 126}$*/TM3, Ser* females, 65% (n=14) and 75% (*n*=36) (Figure 3.1) of the oocytes have progressed to metaphase I at stage 11 and early stage 13, respectively. This number increased to 81.3% (*n*= 16) by mid stage 13 (Figure 3.1). My data thus confirm that *Mtrm*$^{\Delta 126}$ heterozygotes undergo nuclear envelope breakdown precociously as reported (Xiang et al., 2007). Interestingly, I observed that the precocious release from prophase I that I saw in *Mtrm*$^{\Delta 126}$*/TM3, Ser* females was dominantly suppressed by *endos*^{00003} (Figure 3.1). In *Mtrm*$^{\Delta 126}$ +/+ *endos*^{00003} double heterozygous females, all of the early stage 11 egg chambers were still arrested at prophase I (n=15) (65% of *Mtrm*$^{\Delta 126}$*/TM3, Ser* females have entered metaphase I at this early stage), and only1.8% (*n*=113) of the early stage 13 oocytes (vs. 75% in *Mtrm*$^{\Delta 126}$*/TM3, Ser*) or 19.5% (*n*=36) of the mid stage 13 oocytes (vs. 81.3% in *Mtrm*$^{\Delta 126}$*/TM3, Ser)* progressed to metaphase I. This suppression is very similar to the effect observed when one copy of *polo* is removed (Xiang et al., 2007) and thus suggests that the dosage of *endos* influences Polo activity.

Achiasmate fourth chromosome misorientation defects are enhanced in *Mtrm$^{\Delta 126}$* +/+ *endos^{00003}* double heterozygotes

Previous studies with *Mtrm$^{\Delta 126}$*/+ females showed that the non-exchange or achiasmate fourth chromosomes are often found, instead of at opposite poles, to be positioned toward the same pole (examples in Figure 3.2B-C), a phenotype that leads to the high frequency of achiasmate chromosome non-disjunction (Xiang et al., 2007). Based on the known role of heterochromatin pairing in promoting the correct position of these chromosomes on the metaphase plate, it was hypothesized that *Mtrm* function is required for heterochromatic pairing. As mentioned in this chapter's introduction, a cause and effect connection between the precocious nuclear envelope breakdown and the misorientation of fourth chromosome defects was not clearly demonstrated by the reported studies with *Matrimony*.

Because I observed restoration of the timing of nuclear envelope breakdown in *Mtrm$^{\Delta 126}$* +/+ *endos^{00003}* double heterozygotes, I was interested in determining whether these genes also dominantly interact to regulate the orientation of the fourth chromosomes. To score this phenotype, I stained oocytes with DAPI. For the purpose of my studies, I defined this phenotype as "Misoriented Metaphase I" (refered to as Misoriented MI). As indicated in Chapters I and II, the fourth chromosomes can be recognized based on size and position on the metaphase plate. When analyzing wild-type control and *endos^{00003}/TM3, Sb^{e}* stage 14 oocytes, I find that no oocytes show misoriented MI (Figure 3.2D). Consistent with previous observations (Xiang et al., 2007), I identify 32% of *Mtrm$^{\Delta 126}$*/+ stage 14 oocytes with misoriented fourth chromosomes (Figure 3.2B,D). Surprisingly, the amount of *Mtrm$^{\Delta 126}$* +/+ *endos^{00003}* oocytes displaying misoriented MI at this stage is doubled (from 32% to 62.5%) (Figure 3.2C-D). My

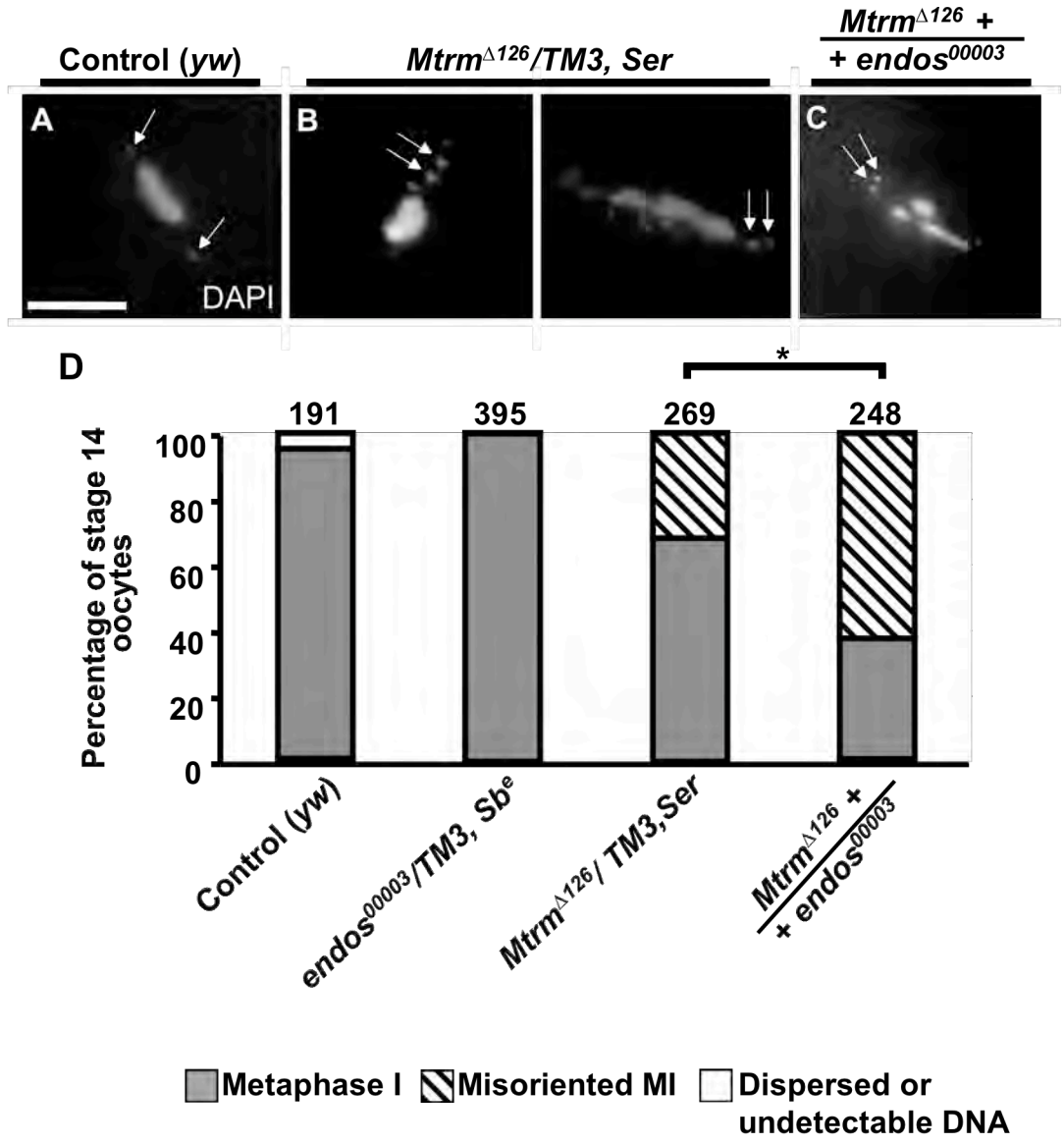

Figure 3.2. *endos*^{00003} dominantly enhances the fourth achiasmate chromosome misorientation defects of *Mtrm*$^{\Delta 126}$*/TM3, Ser* heterozygotes. (A) Control stage 14 oocyte showing normal metaphase I DNA morphology with fourth chromosomes (arrows) positioned at opposite poles. (B-C) Examples of *Mtrm*$^{\Delta 126}$*/TM3, Ser* heterozygous or *Mtrm*$^{\Delta 126}$ *+/+ endos*^{00003} double heterozygous stage 14 oocytes displaying "misoriented Metaphase I" DNA morphology. We define misoriented Metaphase I as when the fourth achiasmate chromosomes are not properly oriented at opposite poles. (D) Percentage of oocytes in metaphase I, or showing misoriented Metaphase I or fragmented or undetectable DNA. Numbers above bars represent the total number of stage 14 oocytes analyzed. Scale bar, 10 μm. Asterisk, $P<0.001$.

findings showing the opposite impact of *endos*^{00003} heterozygosity on the timing of nuclear envelope breakdown and on the fourth chromosome orientation in *Mtrm*$^{\Delta 126}$/+ females demonstrate that these two meiotic defects are not causally related and happen independently from each other in *Mtrm*$^{\Delta 126}$/+ females. In addition, my data is consistent with *endos* controlling at least two different steps in female meiosis through different interactions with *Mtrm* at each of these steps.

Endos regulates Mtrm protein levels

Because I observed an enhancement of the *Mtrm/+* fourth chromosome orientation defects by halving *endos*' dosage, I was interested in determining whether overall Mtrm levels were affected in *endos* mutants. To address this question, I collected egg chambers at different developmental stages and analyzed Mtrm expression by Western blot analysis using anti-Mtrm antibodies previously characterized in Xiang et al. (2007) (Figure 3.3). Control females show low levels of Mtrm from germarium to stage 12 of oogenesis. At stage 13, Mtrm protein levels begin to rise and reach their highest levels at stage 14. These results differ from the immunofluorescence studies by Xiang et al (2007) in which they reported that Mtrm is greatly reduced at stage 13. I have found, however, that immunofluorescence signals can only be detected following dechorionation of oocytes from stage 12 to stage 14 to allow antibody penetration. Thus, this might explain the difference between my results, where Mtrm detection was performed by Western blot analysis, and Xiang et al. (2007) immunofluorescence results. My findings show, however, that the current model that proposes that degradation of *Mtrm* triggers meiotic maturation is incorrect. When I analyze *endos*^{00003} ovaries, I observe relatively

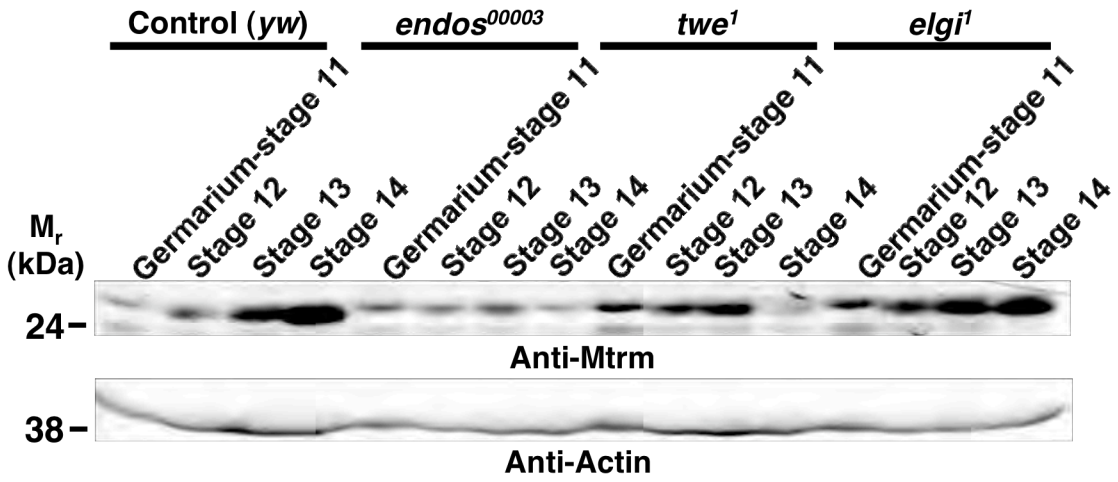

Figure 3.3. Mtrm levels are strongly reduced in *endos*^{00003} mutant stage 13 and 14 oocytes. Mtrm Western analysis of egg chambers at different developmental stages. Wild-type ovarioles show relatively low Mtrm expression from germarium to stage 12, increased Mtrm expression at stages 13 and highest expression at stage 14. *elgi*^{1} mutants show slightly elevated Mtrm levels at earlier stages (germarium to stage 12) but comparable to wild-type levels at stages 13 and 14. *twine*^{1} ovarioles show dramatic reduction of Mtrm only at stage 14. *endos*^{00003} ovarioles display relatively normal levels of Mtrm at early stages, but strong reduction at stages 13 and 14. One-hundred egg chambers or oocytes per lane. Actin was used as a loading control.

normal levels of Mtrm expression at early stages (germarium to stage 12) when compared to control females. They show, however, drastic reduction in Mtrm levels at stages 13 and 14, suggesting that Endos controls Mtrm levels at these later stages.

In addition to the increase in Mtrm expression in control *yw* females as development proceeds, I also observe a downshift in Mtrm mobility (Figure 3.3), potentially corresponding to dephosphorylation at stage 14. Although their roles have not been tested yet, seven phosphorylation sites have been identified in Mtrm by multidimensional protein identification technology (MudPIT) mass spectrometry (Xiang et al., 2007). Interestingly, $endos^{00003}$ stage 14 oocytes do not show the shift in Mtrm mobility that we observe in control oocytes. Thus, these findings suggest that Mtrm phosphorylation at stage 14 may promote Mtrm instability and that Endos controls Mtrm stability by influencing either its phosphorylation status or other factors that specifically degrade the phosphorylated form of Mtrm.

$Mtrm^{\Delta 126}$ +/+ $endos^{00003}$ females are almost completely sterile

While analyzing the phenotypes mentioned above in $Mtrm^{\Delta 126}$ +/+ $endos^{00003}$ double heterozygotes, I noticed that these double heterozygous females were virtually sterile. To better quantify fertility in these females, I collected 0-20 minute fertilized eggs from females of control or experimental genotypes that had been crossed to control *yw* males and measured hatch rates (Figure 3.4). Whereas the hatch rate in control wild-type females is 93% (*n*=450), $endos^{00003}$*/TM3,* Sb^{e} and $Mtrm^{\Delta 126}$*/TM3, Ser* single heterozygous females show 64% (*n*=450) and 38% (*n*=450) hatch rates, respectively. Because I cannot rule out a potential effect of balancer chromosomes on fertility, I might be

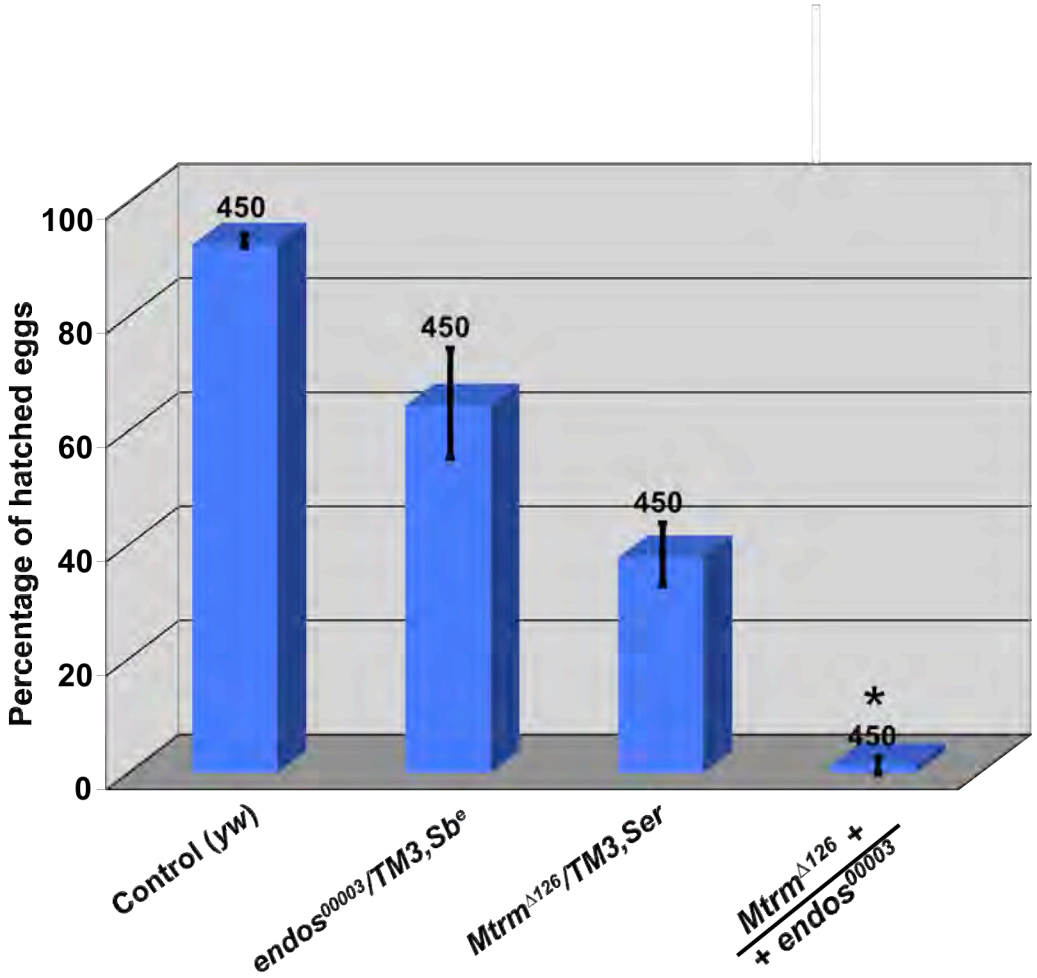

Figure 3.4. ***Mtrm*$^{\Delta 126}$*+/+ endos*^{00003} **double heterozygotes display strong female sterility.** Hatch rate quantification. Numbers above bars represent the number of eggs counted. Asterisk, $P<0.001$.

underestimating these hatch rates. Nevertheless, when I measure hatch rates in *Mtrm*$^{\Delta 126}$ +/+ *endos*^{00003} females, I find that only 1.3% of the laid eggs hatch. These findings further add to the strong dominant genetic interactions between *endos* and *Mtrm* that I observe for the misorientation of fourth chromosomes. In addition, these results are consistent with the model that the strong sterility of *endos* and *Mtrm* double heterozygotes is likely due to non-disjunction defects in meiosis I and/or defects in early embryogenesis. To distinguish between these two possibilities and better understand the causes of the strong sterility, I will analyze DNA and spindles in fertilized (to examine embryos that entered the embryonic mitoses) and unfertilized eggs (to measure the number of oocytes that completed meiosis) derived from *Mtrm*$^{\Delta 126}$ +/+ *endos*^{00003} females as well as from single heterozygous mothers.

Phosphorylation of Endos at a highly conserved site is not required for meiosis or early embryogenesis

The Endos protein shares a putative cAMP-dependent phosphorylation consensus (**R**KX**S**) site with human, mouse, worm, and yeast homologs (Chapter I). Indeed, *in vitro* evidence has shown that this site is phosphorylated by PKA in recombinant mammalian *α-endosulfine* or in response to forskolin treatment in brain rat striatal slices (Dulubova et al., 2001). In *Drosophila*, we observe a downshift in Dendos mobility from stage 13 (data not shown) to stage 14 (Figure 3.5), roughly corresponding to the transition point where the oocyte undergoes meiotic maturation and progresses into metaphase I. Thus, it is reasonable to hypothesize that changes in phosphorylation status may dictate Endos' functions in the cell cycle. To test whether this evolutionarily conserved phosphorylation site is required for Endos regulation, our laboratory generated transgenic lines carrying a

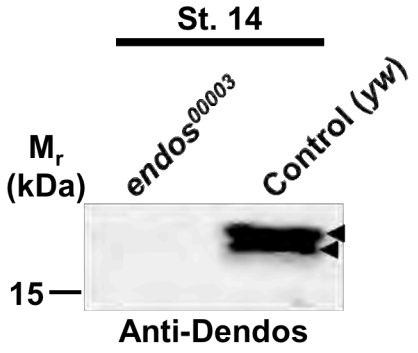

Figure 3.5. Two Endos forms with distinct electrophoretic mobility are observed at stage 14. Endos Western blot analysis of stage 14 oocytes. The lower band may represent dephosphorylation of the conserved PKA phosphorylation site. One twentieth of one pair of ovaries from control *yw* or *endos*^{00003} females were loaded per lane. Arrowheads point to the two Endos migrating bands.

Table 3.1. Rescue quantification in phosphorylation site mutant transgenic lines

***Endos*^{S107A} transgenic lines**	**Chromosome**	**Expression levels**	**Rescue**
UASp-endos$^{S107A\ \#71}$*/CyO*	**II**	**n.d.**	**yes**
UASp-endos$^{S107A\ \#75}$*/CyO*	**II**	**medium**	**yes**
UASp-endos$^{S107A\ \#3}$*/FM 7*	**X**	**low**	**no**
***Endos*^{S107D} transgenic lines**			
UASp-endos$^{S107D\ \#13}$*/FM 7*	**X**	**medium**	**partial**
UASp-endos$^{S107D\ \#19}$*/CyO*	**II**	**n.d.**	**n.d.**
UASp-endos$^{S107D\ \#18}$*/CyO*	**II**	**n.d.**	**n.d.**
UASp-endos$^{S107D\ \#15}$*/CyO*	**II**	**n.d.**	**n.d.**
UASp-endos$^{S107D\ \#32}$*/CyO*	**II**	**n.d.**	**n.d.**

n.d, non determined

non-phosphorylatable (S107A) mutation (Table 3.1) (Research Technician Li Zhang built the construct and generated the transgenic lines.) To determine if Endos^{S107A} was stable and properly expressed, I used *nanos-GAL4::VP16* to drive expression of the *UASpI-endos^{S107A}* transgenes in the germline of *endos^{00003}* mutants and performed Western blot analysis. In *S107A* transgenic lines, I could readily detect the Endos mutant protein although just at slightly lower levels than those of the Endos^{WT} used for the rescue experiments in Chapter II (Figure 3.6). These results suggested that the Endos mutant protein was not grossly misfolded/unstable. I then analyzed oocyte meiotic maturation and metaphase I as well as fertility in rescue experiments (Figure 3.7, Table 3.2). To my surprise, Endos^{S107A} partially rescued meiotic maturation, progression into metaphase I (Figure 3.7B, Table 3.2), and sterility in *endos* mutants. I obtained similar results with two independent transgenic lines (#75 and #71). These findings argue that phosphorylation at the RKXS site is not absolutely required for *endos'* meiotic functions.

Dephosphorylation of Endos may be required for correct fourth chromosome orientation

We were also interested in determining the effect of a phosphomimetic (S107D) mutation on the *endos'* mutant phenotype. Therefore, we generated *UASpI-endos^{S107D}* transgenic lines. I drove the expression of the Endos^{S107D} transgene in the germline of *endos^{00003}* females using the *nanos::VP16-Gal4 driver* and confirmed its expression and protein stability. Endos^{S107D} protein partially rescued meiotic maturation and metaphase I, but fertility was not restored in *endos* mutants. When analyzing mid-stage 13 oocytes expressing Endos^{S107D}, I found that, in addition to normal metaphase I DNA structures (3.9%), 5.9% had misoriented Metaphase I DNA morphology (Figure 3.7C-D, Table 3.2),

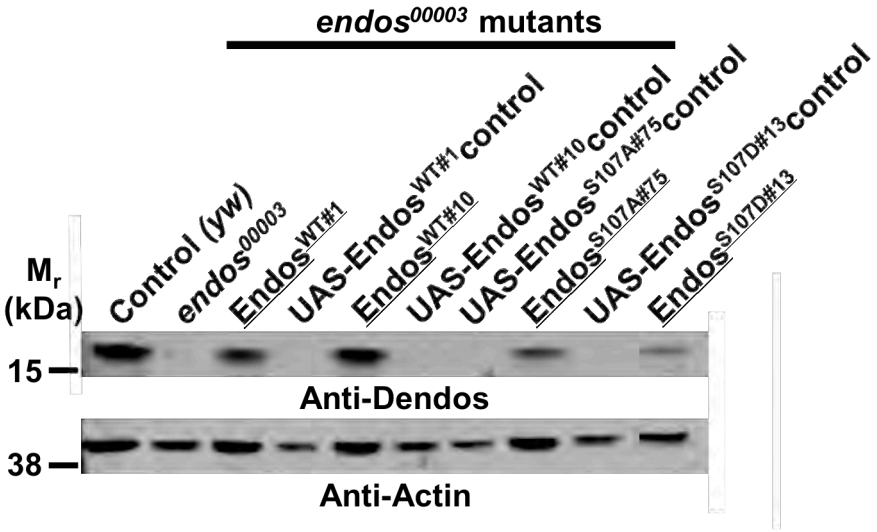

Figure 3.6. Expression of Endos^{WT} or Endos^{S107A} and Endos^{S107D} proteins in *endos^{00003}* mutant background. Western blot analysis of germline expression of endogenous Endos (in wild-type and *endos^{00003}* backgrounds, first two lanes) wild-type Endos^{WT}(#10 and #1), non-phosphorylatable Endos^{S107A} (#71 and #75), and phosphomimetic Endos^{S107D} (#13) in the *endos^{00003}* background. One twentieth of one pair of ovaries per genotype were loaded per lane. Actin was used as a loading control.

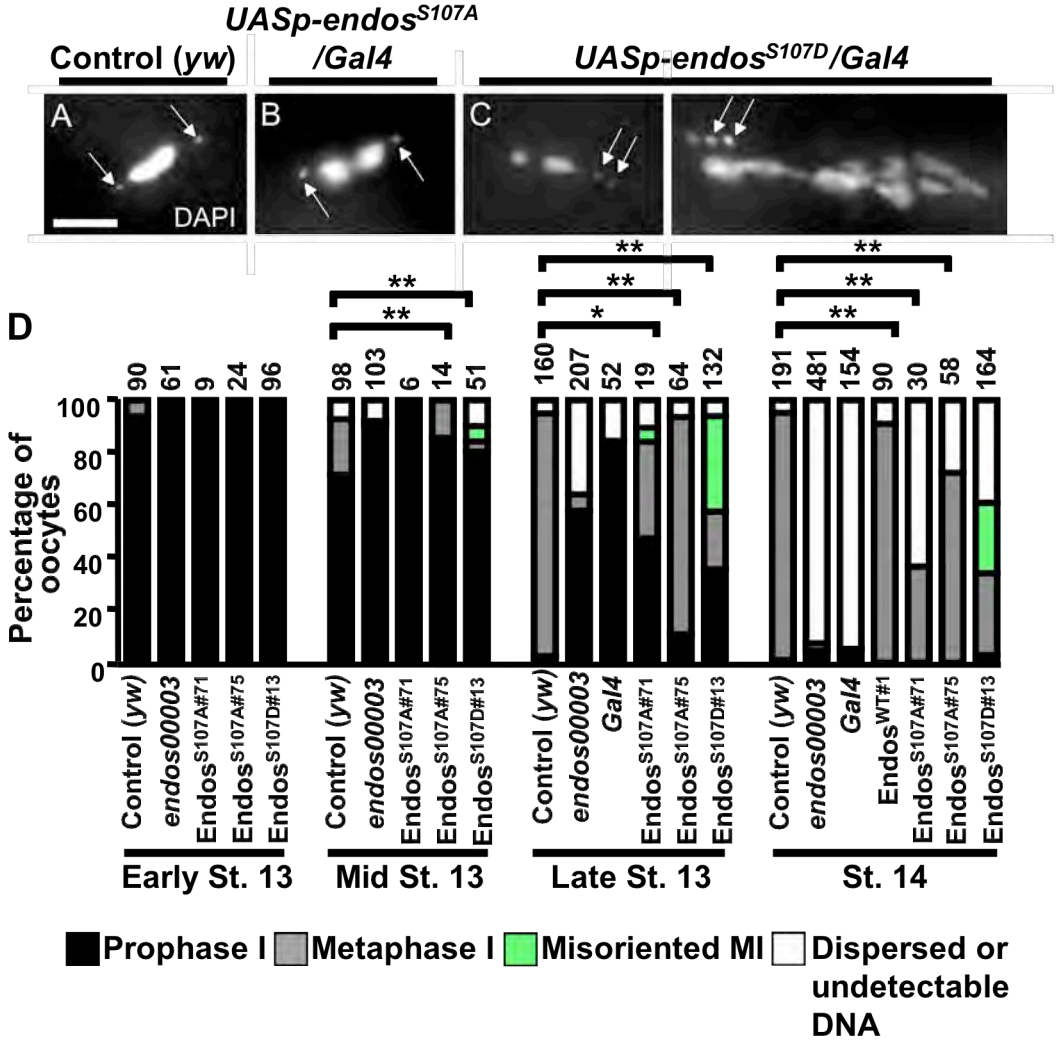

Figure 3.7. *endos*^{S107D} mutants show misorientation of achiasmate fourth chromosomes whereas *endos*^{S107A} mutants show rescue of all *endos* meiotic defects. (A) *yw* control stage 14 oocyte showing normal metaphase I DNA morphology with fourth chromosomes (arrows) positioned at opposite poles. (B) Endos^{S107A}-expressing stage 14 oocyte showing rescue of metaphase I DNA morphology. (C) Endos^{S107D}-expressing stage 14 oocytes displaying misorientation of the fourth chromosomes. (D) Percentage of oocytes in prophase I and metaphase I, or showing misoriented MI or abnormal DNA morphology (dispersed or undetectable DNA). Numbers in parentheses represent the total number of stage 13 and 14 oocytes analyzed. Scale bar, 5 μm. Asterisks, ** P<0.001, * P<0.05.

Table 3.2: Quantification of Endos^{S107A}- and Endos^{S107D}- expressing oocytes arrested at prophase and metaphase of meiosis I

Strain	Percentage of oocytes															
	Early stage 13[b]				Mid stage 13[b]				Late stage 13[b]				Stage 14[b]			
	PI[c]	MI[c]	MOI[c]	Ab[c]	PI[c]	MI[c]	MOI[c]	Ab[c]	PI[c]	MI[c]	MOI[c]	Ab[c]	PI[c]	MI[c]	MOI[c]	Ab[c]
Control[d]	94	6	0	0	71	21	0	7.1	2.5	93	0	5	1	94	0	4.7
		(90)				(98)				(160)				(191)		
***endos*^{00003}**	100	0	0	0	90	1.9	0	7.8	58	6.3	0	36	4.6	2.9	0	93
		(61)				(103)				(207)				(481)		
Control *UAS-S107A#71*	100	0	0	0	100	0	0	0	77	0	0	23	7	1	0	92
		(42)				(21)				(83)				(112)		
Control *UAS-S107A#75*	100	0	0	0	100	0	0	0	58	0	0	42	6	0	0	94
		(22)				(9)				(31)				(50)		
Control *UAS-S107D#13*	100	0	0	0	100	0	0	0	69	2	0	29	3	1	1	95
		(54)				(30)				(84)				(230)		
Control *Gal4*	n.d.[f]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	85	0	0	15	5	0	0	95
										(52)				(154)		
Endos *UAS-WT #1/Gal4*	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0	91	0	9
														(90)		
Endos *UAS-S107A#71/Gal4*	100	0	0	0	100	0	0	0	47	37	5	11	0	37	0	63
		(9)				(6)				(19)				(30)		
Endos *UAS-S107A#75/Gal4*	100	0	0	0	86	14	0	0	11	83	0	6	0	72	0	28
		(24)				(14)				(64)				(58)		
Endos *UAS-S107D#13/Gal4*	100	0	0	0	80	4	6	10	36	22	36	6	3	31	27	39
		(96)				(51)				(132)				(164)		

[a]Experiments were performed at room temperature. [b]Stage 13 oocytes were categorized as early, mid and late according to the number of nurse cell nuclei present: Early stage 13 egg chambers have 11-13 nurse cell nuclei, mid stage 13 egg chambers have 6-10 nurse cell nuclei, and late stage 13 egg chambers have 1-5 nurse cell nuclei. [c]PI, prophase I arrest; MI, metaphase I arrest; MOI, misoriented MI; Ab, abnormal DNA morphology. [d]*yw* females were used as the wild-type control. [e]The number of oocytes analyzed is shown in parentheses. [f]n.d., non determined.

indicating that the fourth chromosomes were not properly oriented on opposite poles on the metaphase plate. At late stage 13, 22% of the Endos^{S107D}-expressing oocytes had normal metaphase I, whereas 36.4% had misoriented Metaphase I. By stage 14, the percentage of oocytes showing normal and misoriented Metaphase I was 31.1% and 26.8%, respectively. I will test the remaining *UASpI-endos^{S107D}* transgenic lines in the future. My Endos^{S107D} analysis suggest that Endos dephosphorylation may be required to maintain heterochromatin pairing and thus to promote the proper orientation of the fourth chromosomes at metaphase I.

Females expressing the Endos^{S107D} mutation are completely sterile despite the presence of normal metaphase I DNA structures

Although I observed some percentage of normal metaphase I DNA structures in *endos^{00003}* females expressing the Endos^{S107D} protein, these females were still completely sterile. Therefore, I hypothesized that, in addition to non-disjunction (caused by the misoriented MI defect), embryonic developmental defects may help account for the sterility of these Endos^{S107D}-expressing females. To begin to test this hypothesis, I analyzed a small fraction (*n*=32) of 0-20-minute embryos derived from mothers expressing Endos^{S107D}. None of the embryos showed normal mitoses (Figure 3.8B-B"). Instead, I observed a range of phenotypes. For example, embryos showed abnormal metaphase I spindles (29%) (Figure 3.8B'), mitotic spindles with misoriented/missing centrosomes (i.e. centrosomes at one side only) (3.2%) (Figure 3.8B"), or metaphase II-like spindles attached at one pole (Figure 3.8C'') or in different focal planes (data not shown). The remaining embryos (54.8%) showed some of the *endos*-like embryonic phenotypes illustrated in Chapter II, which might be indicative of incomplete rescue of

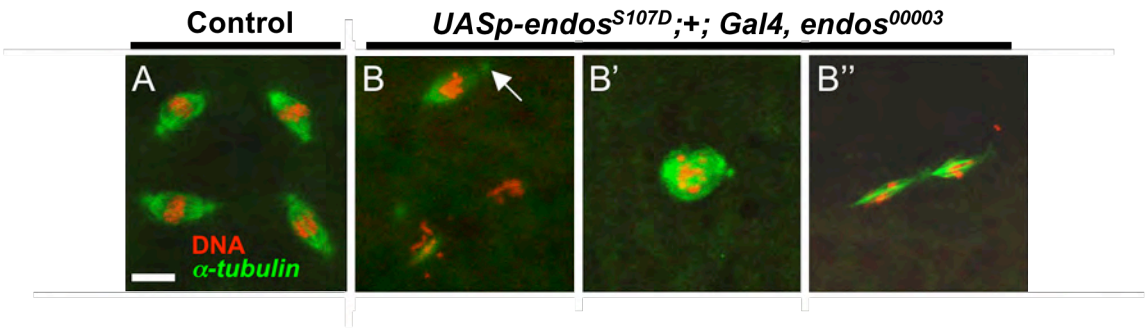

Figure 3.8. Spindle defects are observed in embryos from *endos*^{00003} females expressing the *endos*^{S107D} mutation. (A-B'') Embryos from a 20-minute collection stained with propidium iodide to label DNA (red) and with anti-α-tubulin to label microtubules (green). (A) *yw* control embryos exhibit embryonic mitoses with normal spindles. (B-B'') *UASp-endos*$^{S107D\#13}$*/Gal4*-derived embryos showing a variety of spindle defects, including single mitotic spindles lacking one centrosome (arrow points to position of the centrosome) (3.2%) (B), single metaphase I abnormal spindles (29%) (B'), or two metaphase II-like tandem spindles (13%) (B"). The remaining oocytes (54.8%) displayed *endos*-like phenotypes indicative of incomplete rescue by the transgene. Scale bar, 10 μm. Asterisks, $P<0.001$.

meiosis by the transgene or a role of *endos* in early embryonic mitoses. In contrast to what occurs in many other species, *Drosophila* oocytes complete meiosis independently of fertilization. Thus, I can quantify completion of meiosis by looking for the presence of the four meiotic products (which often fuse and form a rosette structure) in unfertilized eggs. Because in these studies I used fertilized eggs, which entered the embryonic syncytial mitoses, I cannot yet definitely conclude whether or not meiosis was completed normally in these embryos. Nevertheless, my data suggest that blocking Endos dephosphorylation may lead to defects in meiosis I and/or II, and possibly, defects in embryonic mitoses.

Discussion

My findings show, for the first time, that *endos* and *Mtrm* genetically interact during oogenesis. I find that the removal of one copy of $endos^{00003}$ in $Mtrm^{\Delta 126}$*/TM3, Ser* females can suppress the premature meiotic maturation at the same time that it enhances the fourth chromosome misorientation defects. These data therefore suggest that the defects in fourth chromosome orientation and non-disjunction are not simply a consequence of the precocious meiotic maturation of *Mtrm/+* oocytes. Thus, I conclude that *endos* and *Mtrm* interact differently to control the timing of meiotic maturation versus the fourth chromosome orientation during metaphase I in *Drosophila* females.

Xiang et al. (2007) base their model for the role of Mtrm in regulating the timing of nuclear envelope breakdown in *Drosophila* oocytes on the balance between Mtrm and Polo levels. According to their model, Mtrm inhibits the Polo proteins that are expressed in the oocyte before the onset of nuclear envelope breakdown (during stages 11 to 12).

By stage 13 (when meiotic maturation occurs), Mtrm is degraded and Polo activates Cdc25 and subsequently initiates the process of nuclear envelope breakdown. My studies show, however, that Mtrm is not degraded and is, in fact, highly expressed in stage 14 oocytes. These results suggest that Mtrm activity is not regulated by degradation but potentially by a posttranslational modification such as phosphorylation (see below). I also find that the premature nuclear envelope breakdown seen in *Mtrm$^{\Delta 126}$/TM3, Ser* heterozygous females is significantly suppressed by simultaneous heterozygosity of *endos^{00003}*. This result is very similar to the effect of removal of one copy of *polo*. Thus, one possibility is that Polo levels are reduced in *endos^{00003}/TM3, Sb^{e}* heterozygous females, which could be consistent with the defects we observe in *endos* homozygous mutants. According to this scenario, the reduction of *endos* dosage by half in *Mtrm$^{\Delta 126}$* +/+ *endos^{00003}* females may lead to a decrease in Polo levels, which would in turn compensate for the reduced levels of Mtrm inhibitory proteins in *Mtrm$^{\Delta 126}$*/+ females and thus restore the balance between Mtrm and Polo to normalize the timing of nuclear envelope breakdown in the oocyte.

Another possibility is that Endos suppresses Mtrm via Elgi. I also find that *elgi^{1}* mutants undergo nuclear envelope breakdown prematurely and have hypothesized in Chapter II that Elgi-mediated modification of Mtrm may promote its binding to Polo. At the onset of nuclear envelope breakdown, Elgi activity may be inhibited by Endos to release Polo inhibition by Mtrm. It is possible that in *Mtrm$^{\Delta 126}$* +/+ *endos^{00003}* double heterozygotes, a reduction of *endos^{00003}* dosage compensates for the reduction in Mtrm levels by decreasing Elgi inhibition and consequently reinforcing the Mtrm-Polo interaction. This model, however, is largely speculative. I should also mention that there

are some major differences between the phenotype of *Mtrm$^{\Delta 126}$/TM3, Ser* and *elgi^{1}*. Although *elgi^{1}* oocytes enter metaphase I at early stage 13, *Mtrm$^{\Delta 126}$/TM3, Ser* oocytes progress into metaphase I as early as in stage 11, suggesting that Elgi would not be absolutely required for the Mtrm-Polo interaction. Moreover, the *elgi^{1}* mutation does not seem to affect the processes following meiotic maturation or fertility, suggesting that it only controls the meiotic maturation timing but not the fourth chromosome alignment on opposite poles. I have not carefully analyzed, however, fourth chromosome alignment in *elgi^{1}* mutants. In contrast, *Mtrm$^{\Delta 126}$/TM3, Ser* females have a high incidence of misorientation of the fourth chromosomes and non-disjunction, and also have reduced fertility. Until I experimentally address the role of *elgi* in the control of meiotic maturation timing, its relationship to *Mtrm* will remain unclear.

The mechanism for the increased failure of the non-exchange chromosomes to orient themselves toward opposite poles caused by *endos^{00003}* heterozygosity in a *Mtrm$^{\Delta 126}$* heterozygous background is similarly not clear. Although I have not examined non-disjunction, it is likely that *Mtrm$^{\Delta 126}$* +/+ *endos^{00003}* females also have a high incidence of achiasmate non-disjunction based on previous findings (Xiang et al., 2007). Therefore, I can speculate that *endos* is also playing a key role in heterochromatin pairing of achiasmate chromosomes. Interestingly, it has been estimated that, in humans, non-exchange bivalents account for some of the maternal metaphase I non-disjunction events that lead to trisomies (accounting for 45% of trisomy 21) (reviewed in Koehler and Hassold, 1998). Therefore, what I learn about the mechanism by which *endos* regulates this process could potentially be relevant to humans.

My genetic data together with the Xiang et al. (2007) data suggest a model in which Polo downregulation is required, following meiotic maturation, for normal heterochromatin pairing, orientation and segregation of achiasmate chromosomes. Consistent with a required reduction in Polo activity, overexpression of a *UASp-polo* transgene in a *Mtrm/+* heterozygous background enhances the achiasmate non-disjunction defects observed in *Mtrm/+* heterozygotes and induces sterility. One prediction from my data is that, in addition to *Mtrm*, *endos* may be mediating the downregulation of Polo activity or levels after meiotic maturation at stage 14, although this would be difficult to test given the earlier role of *endos* in regulating Polo stability. Alternatively, *endos* may have a more direct role in promoting fourth chromosome orientation downstream of *Mtrm* and *polo*. A direct role for Polo kinase in homologous chromosome pairing or heterochromatin pairing has not been identified in any system yet. There is evidence from different species, however, in support for a role of cohesins in meiotic chromosome pairing initiation and/or maintenance (McKee, 2004). The clearest examples come from studies in the nematode *C. elegans*. For example, knockdown of *C. elegans recombination* 8 (*rec-8*; encoding the meiotic subunit of the cohesin complex) by RNAi does not impair the initial homologous chromosome pairing at leptotene/zygotene but completely disrupts the maintenance of the homologous pairing during pachytene (Pasierbek et al., 2001). *C. elegans* TIM-1 (encoded by the homolog of *Drosophila timeless* gene, *tim-1*) has been shown to physically interact with the cohesin complex (Chan et al., 2003). Interestingly, similar to *rec-8* mutants, a temperature sensitive mutation in *C. elegans tim-8* also results in homologous chromosome asynapsis in pachytene (Chan et al., 2003). It is then possible to speculate that heterochromatin pairing

might also require the function of cohesins and, considering the known role of Polo kinase as a negative regulator or sister chromatid cohesion, that Polo could be indirectly involved in the initiation and/or maintenance of heterochromatin pairing. It will be interesting to unravel how Polo kinase activity influences heterochromatin pairing.

I also observed drastic effects on Mtrm expression (dramatic reduction of protein levels at stages 13 and 14) and mobility (potential lack of dephosphorylation) by *endos*^{00003} loss-of-function at stage 14. Based on these results, I could also speculate that the enhancement of the fourth chromosome orientation defect in *Mtrm +/+ endos* transheterozygote females is due to the further reduction in Mtrm levels caused by *endos* heterozygosity. Endos could be controlling overall Mtrm protein levels or, alternatively, promoting dephosphorylation of Mtrm at stage 14, a process that might be required to ensure Mtrm stability. To address the alternative hypotheses described above, it will be important to investigate whether Mtrm or Polo levels as well as Mtrm mobility are indeed affected in *endos*^{00003} heterozygotes and whether or not the shift in Mtrm mobility is due to changes in phosphorylation status.

Finally, my Endos phosphorylation site mutant analysis suggests that the failure of Endos to be dephosphorylated at stage 14 induces a phenotype very similar to when *Mtrm* function is disrupted. One possible interpretation for these results is that Endos dephosphorylation is required to directly or indirectly control heterochromatin pairing through Mtrm. One prediction of this model is that females expressing Endos^{S107D} in the germline would have low levels of Mtrm. Because reduction in *polo* levels suppresses this phenotype in *Mtrm*$^{\Delta 126}$/+ females, an alternative prediction is that Polo levels could be improperly increased or mislocalized in Endos^{S107D} females. To further understand the

role of dephosphorylation in Endos' functions, I will have to determine, by immunofluorescence and Western blot analyses, whether the expression of Polo and/or Mtrm is altered in females expressing the Endos^{S107D} protein.

My dominant genetic interaction and mutational data described in this chapter provide insights into the molecular mechanism of action and regulation of Endos during meiosis. Endos seems to regulate two distinct processes during meiosis via its genetic interaction with *Mtrm*. My data suggest that the antagonistic functions of Endos and Mtrm on Polo activity are required to set the correct timing of meiotic maturation. In addition, my data also point to a separate role of Endos in promoting, in cooperation with Mtrm, heterochromatin pairing and subsequent fourth chromosome orientation and proper segregation at the metaphase I-anaphase I transition. This effect seems to be mediated by the inhibition of Polo activity and/or levels. Finally, my findings are consistent with the hypothesis that dephosphorylation of an Endos subpool might be required to control its activity during the process of heterochromatin pairing. Based on these findings, and in combination with my findings in Chapter II, I have proposed a general model to explain the two distinct roles of *endos* during female meiosis (see Chapter V, Figure 5.1A-B).

CHAPTER IV

ENDOS DOES NOT REGULATE INSULIN SIGNALING IN ADULT OVARIES OR INTERACTS GENETICALLY WITH *SUR*

The reader will note that this chapter diverges from previous chapters. The data I present in this chapter were collected during the initial phase of this project to test my original hypothesis that *endos* is a positive regulator of insulin secretion in *Drosophila*, which was based on mammalian models. Only after obtaining evidence against this hypothesis, I explored in detail the role of *endos* in meiosis as described in Chapters II and III.

Introduction

One of the main goals in the Drummond-Barbosa laboratory is to address the question of how adult tissues maintained by stem cells respond to changes in diet using the *Drosophila* ovary as a model system. Egg production in *Drosophila* females is maintained by germline (GSC), somatic (SSC) and escort (ESC) stem cells present in the adult ovary (Spradling, 1993b; Spradling et al., 1997) (see Chapter I). The rates of egg production differ dramatically according to nutritional availability (Drummond-Barbosa and Spradling, 2001). Previous studies demonstrated that food availability influences the ability of egg chambers to undergo vitellogenesis (Giorgi and Deri, 1976). Drummond-Barbosa and Spradling studies showed that both germline and somatic stem cells, as well as their dividing progeny, rapidly adjust proliferation and growth rates in response to dietary changes (Drummond-Barbosa and Spradling, 2001). In addition, they determined

that mutations in *chico*, the *Drosophila* insulin receptor substrate (IRS) homolog, impair the ovarian response to a protein-rich diet (see Chapter I, Figure 1.9B). Thus, these studies identified the insulin pathway as one of the key mediators of this response. Later on, it was shown that *Drosophila* insulin-like peptides (DILPs) made in the brain insulin-producing cells play a crucial role in directly regulating the rates of GSC division and germline cyst development as well as the progression through vitellogenesis (LaFever and Drummond-Barbosa, 2005). In these studies it was also demonstrated that the insulin receptor (InR) is required in the germline to mediate the response to diet.

As I discussed in Chapters I and II, in addition to the dehydration and meiotic maturation defects at stage 14, *endos* mutants have a defective ovarian response to diet (Drummond-Barbosa and Spradling, 2004) (see Chapter I), a phenotype that is very similar to the one observed in the insulin receptor substrate (IRS) or *chico* mutants. In mammals, controversial *in vitro* studies have revealed that α-endosulfine promotes insulin secretion via modulation of the pancreatic K_{ATP} channel subunit, SUR1, or inhibits insulin release by binding to Ca^{2+} channels (see Chapter I). Given the unclear role of mammalian α-endosulfine's function, the lack of *in vivo* evidence to clarify the current discrepancies, and the similarity between the *endos* and *chico* phenotypes, I hypothesized that *endos* was promoting secretion of insulin and decided to test this role *in vivo.* The genetic and molecular evidence that I describe in this chapter, however, suggests that in *Drosophila*, *α-endosulfine* does not regulate the secretion of DILPs and does not appear to act via *Sur* in the germline.

Materials and Methods

RNA extraction and RT-PCR analysis

RNA extraction and RT-PCR analysis were performed as described in Chapter II (See materials and methods) with the following modifications: primers DDB89 (5'-GATGGCTTGGAGTATCTCTGGAC-3') and DDB90 (5'-TTTCATGTCCTTGCCGGTATC-3') were used to detect *Sur* mRNA expression by polymerase chain reaction (PCR) with TaKaRa LA Taq PCR system (TAKARA Bio Inc.), using PCR program (94°C, 5 minutes; 94°C, 30 seconds; 54°C, 30 seconds; 68°C, 50 seconds; 68°C, 10 minutes; 30 cycles). RNA was extracted from adult brain, ovary or remaining carcass.

Meiotic maturation and dehydration analysis

Analysis of stage 14 oocyte meiotic maturation and dehydration were performed as described in Chapter II.

Genomic DNA extraction

Genomic DNA extraction was performed by homogenizing 30 *yw* flies (fed on wet yeast for 2-3 days at room temperature) in 200 µl of Buffer A (100mM Tris-Cl, pH7.5; 100mM EDTA; 100mM NaCl; 0.5% SDS). After homogenization, 800 µl of cold LiCl/KAc solution were added to sample and sample was incubated on ice for 10 minutes at room temperature. Sample was spun for 15 minutes at room temperature and supernatant was transferred to a new microfuge tube. Six hundred µl of isopropanol were

mixed with sample and incubated at room temperature for 5 minutes. After incubation, sample was spun for 15 minutes at room temperature and pellet was washed twice with 70% ethanol. Pellet was speed-vac dried and resuspended in 100 µl of 10mM Tris pH8.0. Ten µl of genomic DNA were used for amplification of *Sur* exon 10 and *Sur* exon 22.

Generation of hairpin constructs

Hairpin constructs without an intron spacer were generated as described (Enerly et al., 2002) (Figure 4.5). To build *pJR5* (*pUASt-5'-Exon10-3'— 3'-Exon10-5')* and *pJR6* (*pUASp-5'-Exon10-3'— 3'-Exon10-5')* hairpin constructs, the *Sur* exon 10 was first amplified from *yw* genomic DNA by PCR as described above using primers DDB64 (5'-GGGAATTCCCGTACTCACACATATTGCCACC-3') and DDB66 (5'-TAAACTATGCGGCCGCTTATGCCAAAACATGTTGCCTG-3') introducing *EcoRI* and *NotI* restriction sites, respectively (underlined sequences). A second amplification was performed using primers DDB65 (5'-GGGGTACCCCGTACTCACACATATTGCCACC-3') and DDB66 (5'-TAAACTATGCGGCCGCTTATGCCAAAACATGTTGCCTG-3') containing *KpnI* and *NotI* restriction sites, respectively. PCR program (94°C, 1 minutes; 60°C 1 minutes; 72°C 1 minutes; 72°C 10 minutes; 30 cycles) was used to generate PCR fragments A (*EcoRI-Sur exon10-NotI*) and B (*KpnI-Sur exon10-NotI*) (~890-bp each). PCR fragments A and B were subcloned into *pCR2.1 TOPO* (Stratagene), sequenced, subsequently excised with *EcoRI-NotI* and *KpnI-NotI* digestions, respectively, and gel purified using QIAquick Spin-Gel extraction Kit (Qiagen). *pUASt* and *pUASp* vectors containing a cDNA insert were digested with *KpnI* and *EcoRI* enzymes and fragments corresponding to each vector

(*pUAST* vector, 9.1Kb; *pUASp* vectors, 9.9 Kb) were cut and gel purified using QIAquick Spin-Gel extraction Kit (Qiagen). Triple ligations using purified PCR products A and B as inserts and linearized *pUASp* or *pUAST* vectors were performed in the presence of T4 DNA ligase (NEB) in a water/ice bath overnight. Constructs *pJR5* (*pUASt-Sur exon10 hairpin*) and *pJR6* (*pUASp-Sur exon10 hairpin*) were confirmed by *EcoRI-NotI* and *KpnI-NotI* double digestions or *KpnI*, *EcoRI* and *NotI* single digestions, and used to generate transgenic lines listed in Table 1 as described in Chapter II.

Sur exon22 hairpin constructs with a *white* intron 2 spacer were generated as described (Lee and Carthew, 2003) (Figure 4.6). The *pJR11* (*pUASp*-5'-*Surexon22-3'—whiteintron2—3'-Surexon22-5')* and *pJR10* (*pUASt*-5'-*Surexon22-3'—whiteintron2—3'-Surexon22-5')* hairpin constructs were generated by amplifying exon 22 from *yw* genomic DNA using primers DDB87 (5'-GCTCTAGAGACTATTTGGGTGGATGCATTG-3') and DDB88 (5'-GCTCTAGAAGCGTTCCAGCTTCCTG-3'), containing an *XbaI* restriction site (underlined sequence), and PCR program (95°C, 1 minutes; 55°C 1 minutes; 72°C 50 seconds; 5 cycles; 95°C 1 minutes; 60°C, 1 minutes; 72°C, 50 seconds; 25 cycles). The resulting 845-bp PCR fragment corresponding to the *Sur* exon22 was purified using the QIAquick PCR purification Kit (Qiagen), subcloned into *pCR2.1 TOPO* (Stratagene) and sequenced. The PCR product was excised from *TOPO* vector by *XbaI* digestion and cloned into *pWIZ* vector (*pUAST* vector containing intron 2 of the *white* gene) that was cut with *AvrII*, gel purified with QIAquick PCR purification Kit (Qiagen), and treated with calf intestinal alkaline phosphatase (Promega) for 30 minutes at 37°C. The resulting *pJR9 (3'-Sur exon22-5'-pWIZ)* construct containing the *Sur* exon22 in the 3'-5'

orientation were confirmed by *EcoRI* and *BstB1* double digestion. *pJR9* was cut with *NheI*, gel purified using Qiaquick Spin-Gel extraction Kit (Qiagen), treated with calf intestinal alkaline phosphatase (Promega) for 30 minutes at 37°C and ligated using T4 DNA ligase to *Sur* exon22 fragment cut with *XbaI.* The *pJR10* (*pWIZ-Sur exon22 wintron2* hairpin) construct that had the *Sur* exon22 in the 5'-3' orientation was confirmed by *Bst B1* digestion. *pJR10* was digested with *XbaI* and *EcoRI* and the *EcoRI-3'-Sur exon22-5'-pWIZ* -5'-Sur exon22-*3'-XbaI* fragment was excised, gel purified as described above and subcloned into *pUASp* cut with *EcoRI* and *XbaI* to generate *pJR11* (*pUASp*-Sur exon22 *wintron2* hairpin). *pJR10* and *pJR11* constructs were used to generate transgenic lines described in Table 2 as discussed in Chapter II.

RNAi genotypes

Control genotypes during the RNAi experiments were: (1) *yw; SurE22hp(G)/+;+* (2) *yw; +; SurE22hp(X)/+* (3) *yw; SurE22hp(G)/+; SurE22hp(L)/+* (4) *yw; SurE22hp#24/+; +* and (5) *yw; SurE22hp#10/+; +*. Experimental genotypes were: (1) *yw; SurE22hp(G)/+;actin-Gal4/+* (2) *yw; +; SurE22hp(X)/actin-Gal4* (3) *yw; SurE22hp(G)/+; SurE22hp(L)/actin-Gal4/+* (4) *yw; SurE22hp#24/+; nanos::VP-16-Gal4/+* (5) *yw; SurE22hp#10/+; nanos::VP16-Gal4/+*.

Egg counts

To perform egg counts, 0-2 days old flies of experimental and control genotypes were grown in plastic bottles containing molasses plates with a layer of wet yeast (changed daily). To measure egg production, five pairs of flies per bottle were cultured

and the number of eggs laid was counted every 24 hours in triplicate. Experimental genotype was: (1) *yw;pUASp-endos #1/+;nanos::VP16-Gal4,endos*^{00003}. Control genotypes were: (1) *endos*^{00003}*/TM3Sb*^{e}, (2) *yw;pUASp-endos #1/+;endos*^{00003}, (3) *yw;+; nanos::VP16-Gal4,endos*^{00003}.

Sur in situ hybridization analysis

To generate *Sur* (experimental) and *Oskar* (control) *in situ* probes, the corresponding partial or full length cDNAs in *Sur-pOT2* (SD08664) and *Oskar-pBSK*$^{+}$ vectors were linearized using *BglII* (for *Sur* anti-sense probe) or *AatII* (for *Sur* sense probe) and *HindIII* (for *Oskar* anti-sense probe) or *NotI (*for *Oskar* sense probe) restriction enzymes, respectively. Linearized DNAs were purified by phenol:chloroform extraction and resupended in 10mM Tris, pH8.0. One μg of purified DNA templates were used to generate digoxigenin (DIG) labeled, single stranded RNA probes by *in vitro* transcription using Sp6 (*Sur* anti-sense probe), T7 (*Sur* sense probe) (*Oskar* anti-sense probe) or T3 (*Oskar* sense probe) RNA polymerases and DIG RNA labeling kit (Roche). All RNA probes were size-reduced by addition of 1 μl 0.5M EDTA, 70ul RNAse free water and 10ul 1M Na carbonate pH10.2 and incubation for 30 minutes at 65°C. Following incubation, 6 μl glacial acetic acid, 13 μl 3M NaoAC, 4.5 μl tRNA (9mg/ml) (Sigma) and 280 μl ethanol (EtOH) were added to the probes and probes were stored overnight at -20°C. Samples were spun at maximum speed for 30 minutes at 4°C, rinsed twice with 70% EtOH, air dried, resuspended in 100 μl hybridization buffer (50% formamide (Sigma); 5X SCC; 50 μg/ml heparin (Sigma); 100 μg/ml tRNA (Sigma); 0.1% Tween 20; pH 5.0) and stored at -80°C.

In situ hybridization experiments were perfomed by dissecting *yw* females (as described in Chapter II) fed on wet yeast for 2-3 days at room temperature. Ovaries were fixed by gentle hand rotation in a 50% formaldehyde (FA) (36%) (EM Science)/*n*-heptane (Sigma) mix for 5 minutes at RT; washed twice in methanol, twice in 50% methanol/PBT mix, and twice in PBT for 2 minutes at RT; post-fixed in 4% FA and 1X PBS for 10 minutes at RT; washed 5 times in PBT, one time in 50% PBT/hybridization buffer mix and one time in PBT for 5 minutes at RT. Samples were pre-hybridized for 3 hours at 65°C in hybridization buffer and subsequently incubated with 500 ng/ml of RNA probes at 65°C O/N. After O/N incubation, samples were washed at 65°C for 20 minutes one time with hybridization buffer, one time in 50% hybridization buffer/PBT mix and 4 times in PBT. They were subsequently blocked with 0.5% non-fat dry milk for 1 hr at RT and incubated O/N at 4°C with 7.5 mU/ml Horse Radish Peroxide-anti-DIG-sheep Fab fragment (Roche) and 5% heat inactivated normal sheep serum in PBT. Samples were washed 3 times for 10 minutes in PBT, incubated for 15 minutes at 4°C in 1:50 FITC Tyramide signal amplification diluent (Renaissance), washed 3 times for 5 minutes at RT in PBT and mounted and analyzed as described in Chapter II.

Results

endos does not appear to regulate *Drosophila* insulin-like peptide (DILP) secretion

Taken together, the non-cell autonomous role of *endos* in the response to diet and the similarity of the phenotype with *chico* mutants suggested that perhaps *endos* was controlling DILPs secretion. I reasoned that if *endos* was acting in the brain insulin-

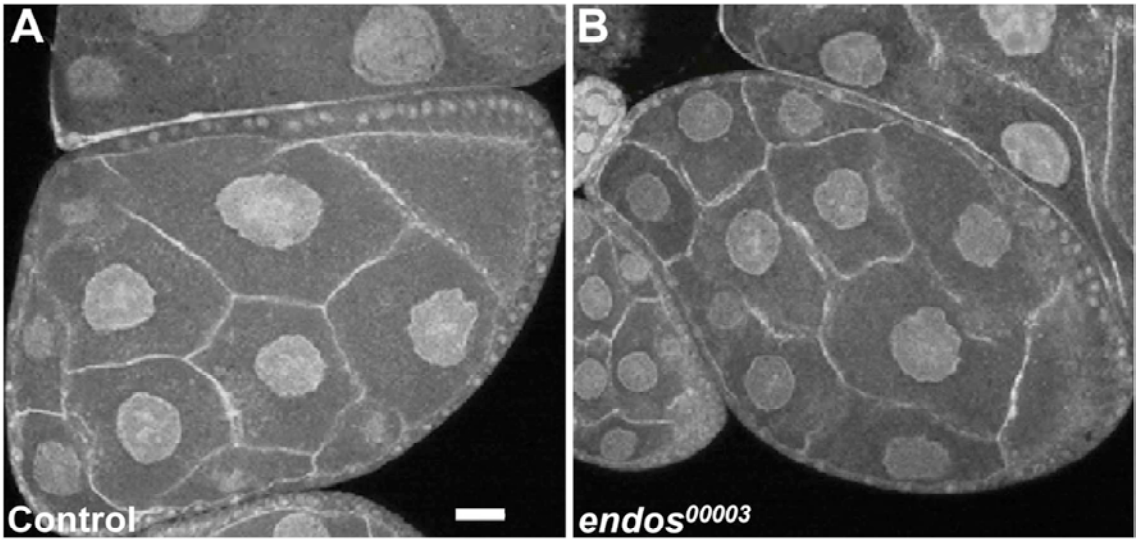

Figure 4.1. *endos* does not appear to regulate insulin signaling in adult ovaries. To test if *endos* mutants have reduced levels of insulin-like peptide secretion, we measured insulin pathway activation in the germline by using the in vivo phosphoinositide 3-kinase (PI3K) kinase tGPH *in vivo* reporter. This reporter contains a fusion of the pleckstrin homology domain of the *Drosophila* GRP1 to GFP. Upon insulin receptor activation, tGPH binds to phosphatidylinositol (3,4,5)-trisphosphate and is recruited to the plasma membrane. The membrane enrichment of tGPH in control (A) and *endos*^{00003} mutant (B) germ cells is comparable, suggesting that *endos* likely does not control insulin-like peptide secretion. A and B are at the same magnification; scale bar, 20 μm.

producing cells to promote the secretion of DILPs, restoring Endos expression specifically to these cells would be sufficient to rescue the low egg production rates that we observed in the mutants. To test this prediction, I drove expression of a *UAS-endos* transgene specifically in these cells using the previously reported *dilp2-Gal4* driver (Rulifson et al., 2002) and measured egg production. Expression levels of all the *UASt-Endos* transgenic lines that I tested were comparable to those in *UASp-Endos* transgenic lines that I used in germline-specific *endos* rescue in Chapter II. Unfortunately, in six out of seven experiments I obtained variable and inconsistent results and, therefore, I could not make any conclusions.

It is not currently possible to detect DILPs circulating in the hemolymph of flies and, therefore, I could not test directly whether insulin secretion was reduced in *endos* mutant flies. Instead, I used tGPH, a phosphoinositide 3-kinase (PI3K) kinase *in vivo* reporter (Britton et al., 2002), to measure insulin receptor signaling (Figure 4.1). This reporter contains a fusion of the pleckstrin homology domain of *Drosophila* GRP1 to GFP. Upon insulin receptor activation, tGPH binds to phosphatidylinositol (3,4,5)-trisphosphate and is recruited to the plasma membrane. This reporter shows reduced enrichment at the membrane in *Drosophila insulin receptor* (*dinr*) mutants (Hsu et al., 2007). In contrast, I did not observe any significant decrease in membrane GFP enrichment in *endos* mutants when compared to control females. Together, these findings suggest that in adult *Drosophila* females, *endos* does not regulate secretion of DILPs.

An alternative possibility is that the reduced rate of oogenesis in *endos* females is an indirect consequence of the meiotic maturation defects. To test this possibility, I drove the same functional *UASp-endos* transgenes, described in Chapter II, in the germline of

endos mutants using the germline-specific *nanosVP16::Gal4* driver and measured the rates of egg production (Figure 4.2). Unfortunately, the *Gal4* driver control (in the *endos* mutant background) also showed high levels of egg production, rendering these experiments uninformative. I also tested other known germline drivers such as *pCOG-Gal4* but their low expression in the germline was not sufficient rescue any of the *endos* meiotic defects.

Sur and *endos* are expressed in a similar set of tissues

Mammalian *in vitro* studies led to the model that *α-endosulfine* was involved in the regulation of insulin secretion (Peyrollier et al., 1996; Virsolvy-Vergine et al., 1992). These studies also identified SUR1 as the target for both sulfonylureas and α-endosulfine binding. I reasoned that, although I did not find a role for *endos* in insulin secretion, it was still a possibility that it could interact with this channel to perform a different biological function. To begin testing this hypothesis, I first analyzed in what tissues *Drosophila Sur* was expressed. Although RT-PCR data had revealed expression in adult and larval stages, detailed analysis of the *Sur* expression pattern had not been done. If Endos and Sur were interacting partners (as the mammalian model suggests), I would expect to find *Sur* expressed in at least some of the tissues where *endos* is expressed (see Chapter I, Figure 1.8). To test this prediction, I examined whether *Sur* and *endo*s had overlapping expression patterns through RT-PCR analysis. I detected *Sur* expression in the embryo (as previously reported) (data not shown), and in the head and ovary, two of the adult tissues where Endos is expressed (Figure 4.3B). Although I performed *in situ* hybridizations in the ovary, *Sur* expression was below the detection levels. To

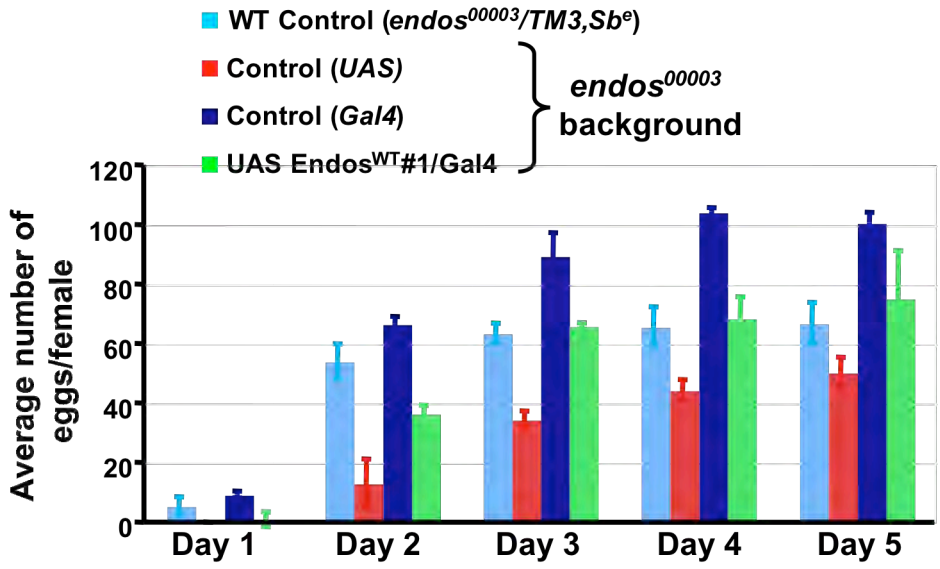

Figure 4.2. Germline-specific expression of Endos rescues the rates of egg production but control Gal4 driver also shows high egg production rates. *endos*^{00003} mutant UAS control (red bars) show low rates of egg production relative to control (*endos*^{00003}*/TM3,Sb*^{e}) (light blue bars). *endos*^{00003} mutant females in which Endos is expressed in the germline (green bars) show significant rescue of the egg production rates. However, the *endos*^{00003} mutant Gal4 control (dark blue bars) show very high rates of egg production. Thus, these data render this experiment uninformative.

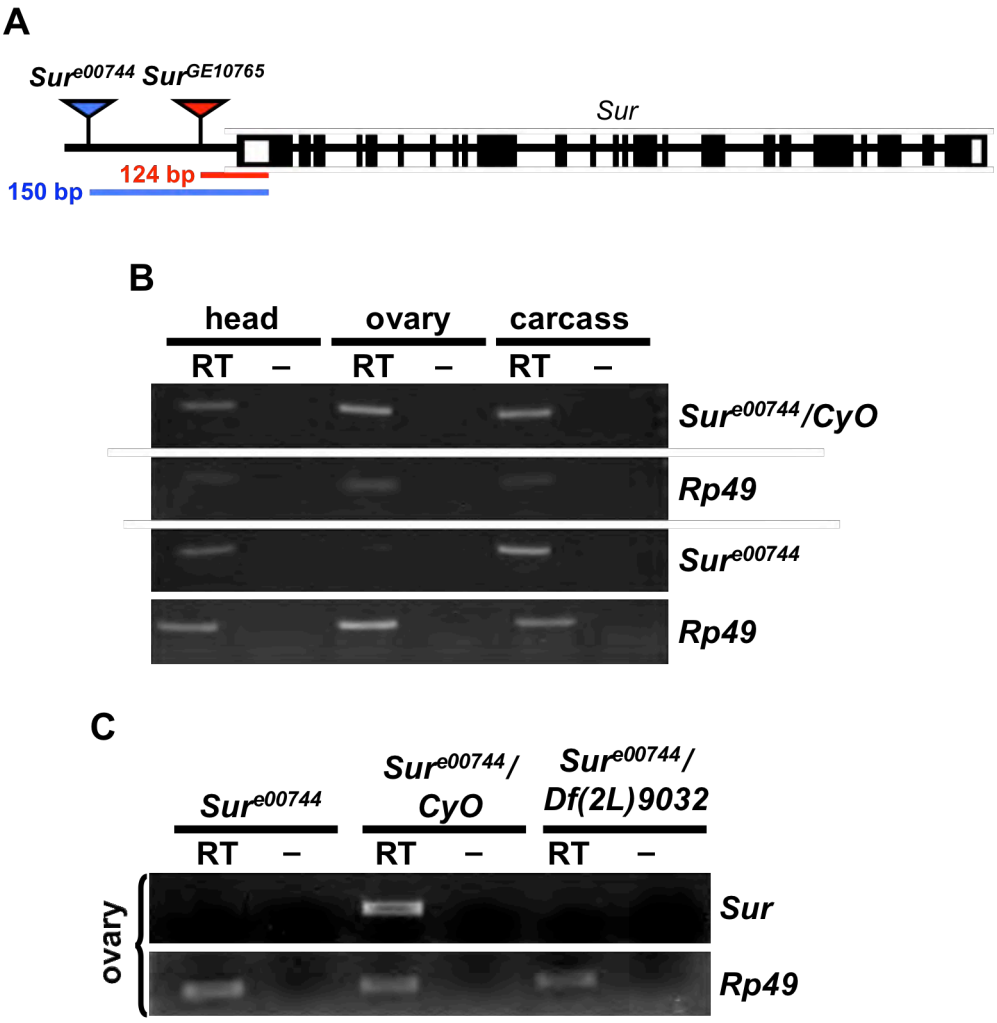

Figure 4.3 The *Sur*^{e00744} insertion severely reduces *Sur* mRNA levels in the ovary. (A) Diagram illustrating position of *pBac* *Sur*^{e00744} and *P* element *Sur*^{GE10765} insertions, respectively, with respect to *Sur* gene, which contains 24 exons. (B) RT-PCR data shows that *Sur* mRNA levels are reduced exclusively in ovaries of *Sur*^{e00744} homozygous flies but not in other adult tissues. *Sur*^{e00744}*/CyO* flies were used as a control. (C) RT-PCR data confirms reduction of *Sur* transcript in ovaries from hemizigous *Sur*^{e00744} over *Df(2L)9032* females. *RP49* was used as loading control.

complement the RT-PCR data and attempt to test for an *in vivo* interaction between Endos and Sur, our laboratory generated four antisera against two different regions of the *Drosophila* Sur protein (Li Zhang, unpublished). Unfortunately, these antibodies could not specifically detect Sur by either immunofluorescence or western blot analysis. Nevertheless, the overlapping expression pattern between *endos* and *Sur* supported the hypothesis that these genes were acting in the same pathway.

RNAi does not efficiently reduce *Sur* transcript levels in germline or somatic tissues

At the time of these experiments, the Sur^{e00744} and $Sur^{GE10765}$ mutational insertions described below were not available. Therefore, I decided to reduce *Sur* mRNA ovarian levels by RNAi to test whether *endos* was acting through *Sur* to promote oocyte dehydration or fertility. I used UAS/Gal4-driven inverted repeats (IR), which are predicted to form double stranded RNA hairpins to conditionally silence gene expression in *Drosophila* in a tissue-specific manner (Celotto and Graveley, 2002; Enerly et al., 2002). I first targeted exon 10 of *Sur* (865 base pairs), which is the largest exon in this gene (24 exons total). To generate the IR transgenes, I built hairpin constructs following the same strategy described by Enerly et al (Enerly et al., 2002) in where *Sur* exon 10 fragments were subcloned in an opposite orientation (5'-Exon10-3'— 3'-Exon10-5') into the *pUAST* (for somatic tissue expression) (Figure 4.4A) and *pUASp* (for germline expression) (Figure 4.4B) vectors. After generating transformant lines (Table 4.1), I ubiquitously drove the expression of *SurExon10*-RNA hairpins by crossing each line to *tubulin-Gal4* and *actin-Gal4* drivers. Progeny expressing the double-stranded RNA hairpin for *Sur* did not show any obvious mutant phenotype.

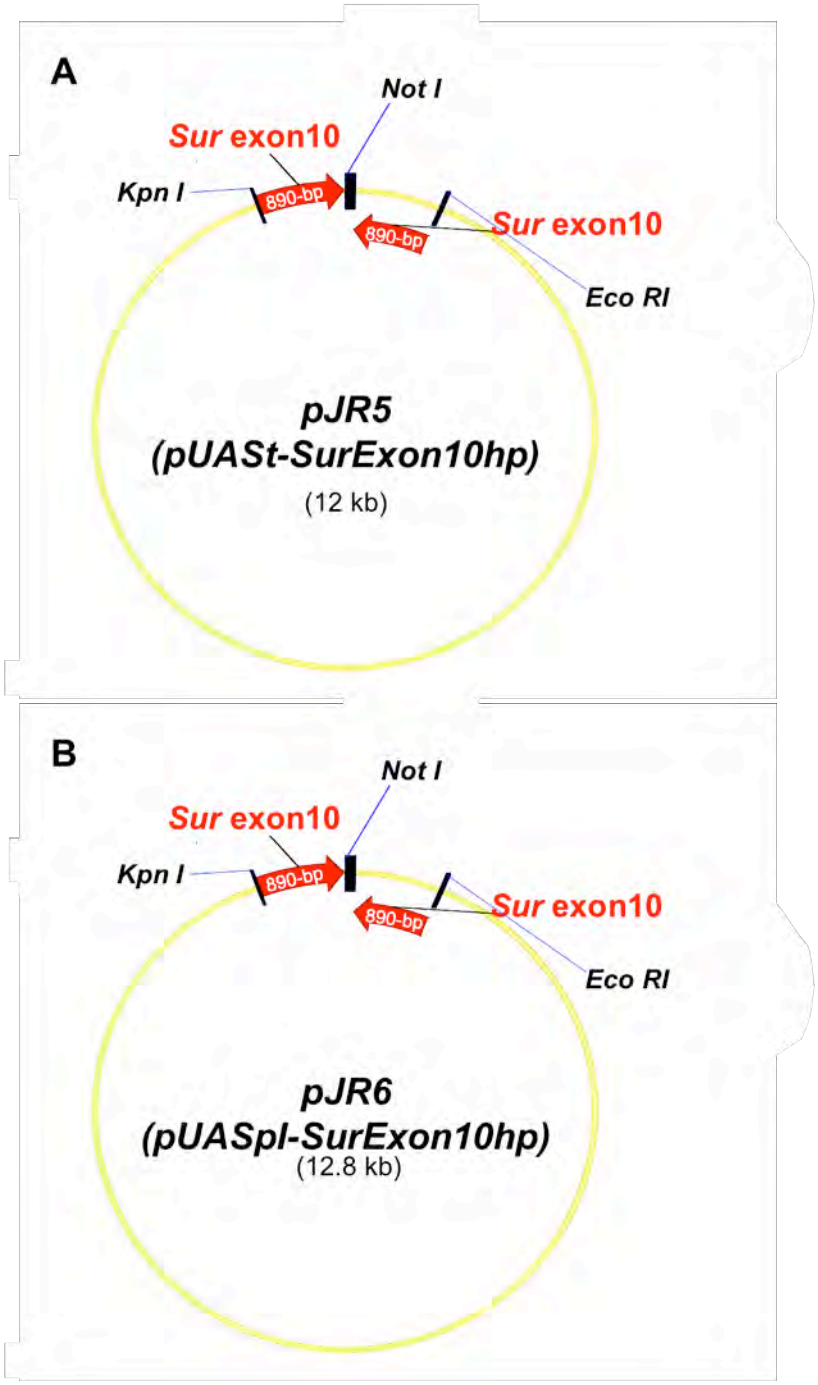

Figure 4.4. *Sur* exon 10 hairpin constructs. *Sur* exon10 inverted repeats were subcloned into *pUASt* (*pJR5*) and into *pUASpI* (*pJR6*) vectors for expression of RNA hairpins in somatic and germline tissues, respectively. Both *UASt* and *UASpI* vectors contain the hsp70 promoter and terminator SV40 (Thummel & Pirrota, 1992), and a mini-*white* gene (provides a selective eye color marker).

To enhance RNAi efficiency, I generated *Surexon22 (*exon 22 is also a large exon in *Sur* gene) inverted repeats but this time cloned them into *pUAST* and *pUASp* vectors containing an intron spacer (second intron of the *white* gene) (Figure 4.5). Addition of an intron spacer to separate the IR sequences has been shown to reduce inconsistent silencing and thereby increase RNAi efficiency in *Drosophila* (Lee and Carthew, 2003). Using a similar strategy mentioned above, I drove expression of the 3'-*Surexon22-5'—whiteintron2—5'-Surexon22-3'* hairpin in somatic tissues and in the germline by crossing several transgenic lines (Table 4.2) to *actin-Gal4* and *nanosVP16::Gal4* drivers, respectively. Regardless of where the *Sur* hairpin was expressed, females did not have any visible phenotype and were fertile. My RT-PCR data showed that the levels of *Sur* in the brain (Figure 4.6A-B) and ovary (Figure 4.6D) were not reduced or eliminated in all the transgenic lines. Finally, recombining more than one transgene was not enough to reduce *Sur* transcript levels (Figure 4.6C).

Sur and *endos* do not genetically interact in the germline

To determine whether *Sur* was involved in *endos*-regulated processes, I obtained two recently available transposable element insertions in the *Sur* gene to study the *Sur* loss-of-function phenotype. Both insertions are homozygous viable. One of these insertions was a *piggyBac* (*pBac*) element insertion (*piggyBac(Rb)Sur*^{e00744}) (referred to as *Sur*^{e00744}) located 150 base pairs upstream of the translational initiation site of the *Sur* gene (Figure 4.3A). In addition to the *pBac* insertion, I also obtained a *P-element* insertion (*GE10765*) (referred to as *Sur*^{GE10765}) in *Sur* located 124 base pairs upstream of

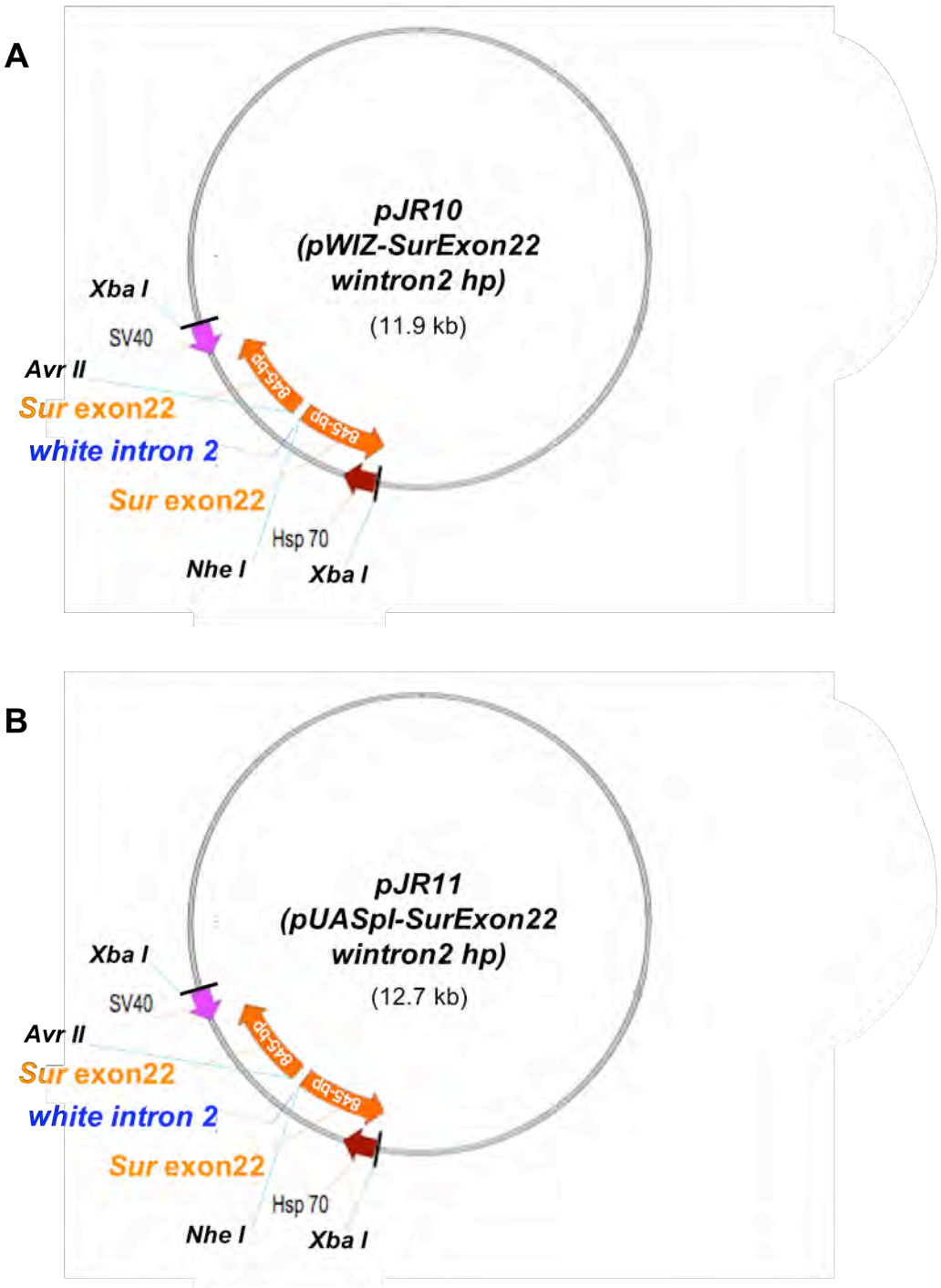

Figure 4.5. *Sur* exon 22 RNAi constructs with *white* intron 2 spacer. *Sur* exon22 inverted repeats were subcloned into *pWIZ* (*pJR10*) and into *pUASpI* (*pJR11*) vectors for expression of RNA hairpins in somatic and germline tissues, respectively. Both *pWIZ* and *UASpI* vectors contain the *hsp70* promoter and SV40 terminator (Thummel & Pirrota, 1992), and a mini-*white* gene (provides a selective eye color marker).

Table 4.1. List of *Sur* hairpin transgenic lines without intron spacer for *Sur* RNAi

***Sur* Exon10 hairpin transgenic lines**		
***UASt* transgenic lines**	**Chromosome**	**Balancer**
pUASt-SurExon10hp (12A)	III	*TM3,Sb*^{e}
pUASt-SurExon10hp (32C)	III	*TM3,Sb*^{e}
pUASt-SurExon10hp (5A)	III	*TM3,Sb*^{e}
pUASt-SurExon10hp (15B)	III	*TM3,Sb*^{e}
pUASt-SurExon10hp (20A)	III	*TM3,Sb*^{e}
pUASt-SurExon10hp (20B)	II	CyO
pUASt-SurExon10hp (22A)	III	*TM3,Sb*^{e}
pUASt-SurExon10hp (28A)	II	CyO
pUASt-SurExon10hp (28C)	III	*TM3,Sb*^{e}
pUASt-SurExon10hp (13B)	II	CyO
pUASt-SurExon10hp (15B)	III	*TM3,Sb*^{e}
pUASt-SurExon10hp (9A)	II	CyO
pUASt-SurExon10hp (9B)	III	*TM3,Sb*^{e}
***UASpI* transgenic lines**		
pUASpI-SurExon10hp (7A)	II	CyO
pUASpI-SurExon10hp (23A)	II	CyO

Table 4.2. List of *Sur* hairpin transgenic lines with intron spacer for *Sur* RNAi

***Sur* Exon22 *white intron 2* hairpin transgenic lines**		
***UASt* transgenic lines**	**Chromosome**	**Balancer**
pUASt-SurExon22 wintron2 hp (X)	III	*TM3,Sb*^{e}
pUASt-SurExon22 wintron2 hp (V)	III	*TM3,Sb*^{e}
pUASt-SurExon22 wintron2 hp (BB)	II	*CyO*
pUASt-SurExon22 wintron2 hp (G)	II	*CyO*
pUASt-SurExon22 wintron2 hp (L)	III	*TM3,Sb*^{e}
***UASpI* transgenic lines**		
pUASpI-SurExon22 wintron2 hp (#24)	II	CyO
pUASpI-SurExon22 wintron2 hp (#10)	II	CyO
pUASpI-SurExon22 wintron2 hp (#12)	III	*TM3,Sb*^{e}
pUASpI-SurExon22 wintron2 hp (#36)	III	*TM3,Sb*^{e}

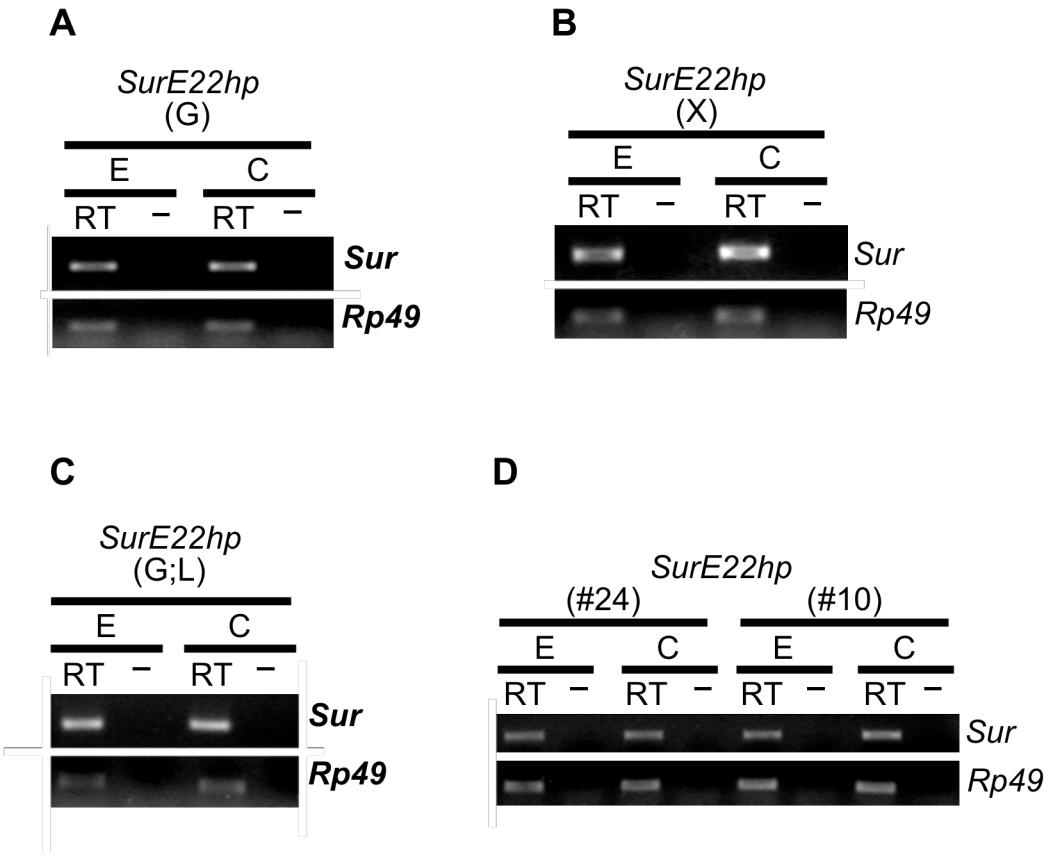

Figure 4.6. RNAi was not an effective approach to disrupt *Sur* mRNA transcript in the ovary or other tissue. Transgenic lines carrying *Sur* exon22 inverted repeats with an intron spacer cloned in *UAST* (A-C) or *pUASpI* (D) vectors were crossed to *actin-Gal4* and *nanosVP16::Gal4* to drive expression of the RNA hairpins in somatic and germline tissues, respectively. (A-B) Brain *Sur* mRNA levels were not reduced in most of the transgenic lines tested (only line G (pannel A) and line X (pannel B) are shown). (C) Recombination of two *pUAST-SurE22hp* transgenic lines (G and L) did not result in *Sur* mRNA elimination or reduction from the brain. (D) Ovary *Sur* mRNA levels were not reduced in all transgenic lines tested (only lines #24 and #10 are shown). E, experimental (RNAi); C, wild-type control. *Rp49* was used as a loading control.

the translational start codon from GenExel, Inc. (Figure 4.3A). To determine if *Sur* expression was disrupted by these insertions, I performed RT-PCR analysis to look for *Sur* mRNA levels. I found that the Sur^{e00744} insertion severely reduces the levels of *Sur* transcripts in the ovary (~95% reduction) but not in the adult heads and carcasses (Figure 4.3B). I observed the same level of reduction when I placed the Sur^{e00744} insertion over deficiency *Df(2L)9032,* which uncovers the *Sur* gene (Figure 4.3C). On the other hand, the $Sur^{GE10765}$ insertion did not disrupt *Sur* expression in any of these tissues (data not shown). With the goal of generating a null *Sur* allele, I crossed the $Sur^{GE10765}$ *P* element to a source of transposase to induce imprecise excisions but this *P* element could not be mobilized, as I failed to recover any excision events after three attempts (approximately 300 flies were screened).

Because Sur^{e00744} females had a strong reduction of *Sur* mRNA levels in the ovary, the tissue where Endos is required for stage 14 dehydration and meiotic maturation (see Chapter II), I tested for Sur^{e00744};$endos^{00003}$ genetic interactions in this tissue and asked whether *Sur* was involved in *endos*-mediated germline processes. Using the mammalian model as a foundation, I hypothesized that the binding of Endos to Sur may be necessary to initiate a cascade of events (similar to the ones proposed for insulin secretion) that would lead to the movement of an aquaporin-like channel (Agre and Kozono, 2003; Fujiyoshi et al., 2002) from vesicles to the plasma membrane to release water in stage 14 oocytes. Alternatively, Sur closing could be necessary to set an osmotic gradient responsible for the loss of water from the stage 14 oocytes. In either case, I would expect that *Sur* loss-of-function in the germline would resemble channel closure and rescue the stage 14 dehydration defects in *endos* mutants. After generating

Sur^{e00744}*;endos*^{00003} double mutants, I observed that only 10.7% (n=1,665) of the stage 14 oocytes were dehydrated, a percentage that was not significantly different from the 4.5% (n=1,246) observed in control *Sur*^{e00744}*/CyO;endos*^{00003} oocytes (*P*>.05). I also analyzed genetic interactions for meiotic maturation, another germline-specific phenotype in *endos* mutants (see Chapter II), and found that the majority of the *Sur*^{e00744}*;endos*^{00003} stage 14 oocytes failed to arrest at Metaphase I (only 1.5% of the oocytes were at Metaphase I; n=195), a phenotype that was similar to what I observed in *endos*^{00003} mutants alone (see Chapter II, Table 2.1), thus suggesting that meiotic maturation was not rescued in the double mutants. Together these findings suggest that *endos* may not regulate dehydration or meiotic maturation in stage 14 oocytes through *Sur*. However, there are alternative interpretations for these results (see Discussion below).

Discussion

Based on the previously reported *endos* mutant analysis data and the mammalian *in vitro* studies, I had hypothesized that *endos* was a positive regulator of insulin secretion in *Drosophila*. My results, however, do not support this hypothesis and suggest that the insulin pathway is not affected in *endos* mutants. My findings showing relatively normal levels of insulin pathway activation in *endos*^{00003} females argue that insulin secretion is not affected in these mutants. Furthermore, the failure of Endos expression in the brain insulin-producing cells to restore normal egg production rates in *endos* mutants further suggest that this defect is not due to a role of *endos* in insulin secretion. It is possible, however, that in addition to the insulin pathway (which is not affected in the mutants), a yet unidentified pathway modulated by *endos* might be controlling the

response to diet in *endos* mutants. Alternatively, Endos might be binding to Sur and controlling the secretion of a different factor (other than DILP) from the brain or the ovary to regulate the ovarian response to diet. Another possibility is that the low rates of egg production, and the inability of *endos* mutant follicle cells to increase their rates of division in reponse to a rich diet are likely a secondary effect of *endos* meiotic maturation defects. The ovary could sense the defects in meiotic maturation and dehydration and reduce the rates of egg production. Obtaining a strong germline driver (with no background defects) to drive sufficient Endos expression in the germline of *endos* mutants and restore normal egg production rates and fertility will help me test whether *endos* functions in the germline, and not in other tissues, for the reponse to diet.

Although I concluded that *endos* does not promote dehydration at stage 14 or induce meiotic maturation via *Sur*, there are alternative explanations for my results. Because the Sur^{e00744} mutation is not a null allele, I cannot rule out the possibility that the low remaining levels of *Sur* activity in the ovary are sufficient to prevent any dehydration rescue in oocytes from *Sur;endos* double mutants. An alternative possibility is that in the ovary *endos* might be involved in promoting stage 14 dehydration by binding to other targets (i.e. Ca^{2+} channel). These hypothesis become less attractive, however, when I consider the interesting and strong connection I consistently observe between defects in meiotic maturation and dehydration (see Chapter II). As mentioned in Chapter II and III, every mutant that I have analyzed with defects in meiotic maturation (i.e. *endos*, *twine*, *cdc2*, and *matrimony*) also displays dehydration and yolk distribution defects. Based on these observations, I favor the hypothesis that dehydration is secondary to the early defects in the meiotic cell cycle of *endos* mutant females. Considering that oocytes

undergo meiotic maturation before dehydration, I could speculate that meiotic maturation serves as a signal to recruit the molecular players required to promote dehydration. Identifying additional players involved in meiotic maturation with defects in dehydration and their role in the *endos* pathway (see Chapter V), will help me elucidate how *endos* is affecting meiotic maturation and dehydration of stage 14 oocytes at the molecular level and how these two processes correlate in *Drosophila*.

My findings in this Chapter differ from the mammalian studies that link α-endosulfine to the physiology of the β-cell in the pancreas and to the control of insulin secretion (Virsolvy et al., 2002; Virsolvy-Vergine et al., 1992). Specifically, the data that I have collected to test the *in vivo* role of *α-endosulfine* in the control of insulin secretion suggest that promotion of DILPs secretion and/or modulation of Sur activity are not part of *endos'* mechanism of action in *Drosophila*. One possible explanation for this discrepancy is that the functions of α-endosulfine are not identical between species. It is possible that higher vertebrates might have developed a newer role for α-endosulfine through evolution. I should emphasize, however, that the mammalian studies were not confirmed by *in vivo* experimentation. Based on the wide range of human *α-endosulfine* expression (Heron et al., 1998) (Chapter I), including the previously unreported ovarian expression I have detected in my studies (Chapter II), I could speculate that *α-endosulfine* plays multiple biological roles. I have identified novel roles of *endos* during meiosis in *Drosophila* (see previous chapters). In fact, the defects in meiotic maturation and progression into metaphase I that I have characterized cause the sterility in *endos* mutants. Thus, as most of this thesis work shows, my focus has been shifted to understanding instead how *endos* regulates the female cell cycle machinery.

CHAPTER V

GENERAL DISCUSSION AND FUTURE DIRECTIONS

The female cell cycle machinery must be strictly regulated during oogenesis to ensure gamete viability and successful embryonic development. Key oocyte meiotic events including arrest at prophase I, maturation and arrest at Metaphase I or II depend on the tight regulation of the activity of Cdk1/Cyclin B. However, the exact molecular mechanisms underlying the control of these meiotic processes via Cdk1/Cyclin B activation or inactivation are still not completely understood. My work described in this thesis identifies *Drosophila α-endosulfine* as a novel cell cycle regulator that has multiple key functions during female meiosis. In this final chapter I provide an overview of my results and conclusions, and discuss my current models for *endos'* meiotic functions, the impact of my findings and, finally, my short- and long-term future directions to further understand *endos*' roles and the control of meiotic maturation.

endos is required for meiotic maturation

As presented in Chapter II, much of my evidence suggests that Cdk1/Cyclin B activation is compromised in *endos* mutants and that this effect is at least partially caused by deregulation of Polo kinase protein levels. Mutations in *endos* cause prolonged prophase I, delayed nuclear envelope breakdown, and impaired progression into metaphase I. These phenotypes are remarkably similar to those observed in mutants of *twine*, the homolog of *Cdc25* (an activator of Cdk1) and *cdc2* (synonymous with *cdk1*).

My data show that *endos*^{00003} females have a strong reduction of Twine and Polo protein (but not mRNA) levels. As discussed earlier, both the stability and the full activation of mammalian Cdc25A require its phosphorylation by Cdk1/Cyclin B, which creates a positive feedback loop that allows Cdc25 to further dephosphorylate and activate Cdk1/Cyclin B (Boutros et al., 2006; Busino et al., 2004; Karlsson-Rosenthal and Millar, 2006). Activation of Cdc25 is also dependent on Polo kinase. *Xenopus* Plx1 phosphorylates and activates Cdc25 to activate Cdk1/Cyclin B and generate Mitotic Protein Monoclonal 2 (MPM2) epitopes (Kumagai and Dunphy, 1996; Qian et al., 1998). In other systems, such as *C. elegans*, disruption of PLK-1 (which also activates Cdc25 in this system) via RNA interference (RNAi) results in defects in nuclear envelope breakdown similar to those caused by RNAi-mediated disruption of the *C. elegans* homolog of Cdk1, NCC-1 (Chase et al., 2000). Based on these data, it is tempting to speculate that the reduction of Twine that we detect in *endos*^{00003} mutants is an indirect consequence of low Polo levels, which could lead to low Cdk1 kinase activity. It remains possible, however, that *endos* controls the stability of these and other key proteins (e.g. Cdc2, Mtrm, see below) via a different mechanism such as, for example, by regulating the activity of the Anaphase Promoting Complex or Cyclosome (APC/C). Furthermore, because I was not able to express high levels of Twine or Polo in *endos* females due to their instability in this background, I could not determine whether reduction of these proteins was responsible for endos' meiotic defects.

endos^{00003} mutant stage 14 oocytes display normal *in vitro* histone H1 Cdk1 kinase activity

Activation of Cdk1/Cyclin B is commonly measured by the *in vitro* phosphorylation of one the Cdk1 substrates, histone H1. As expected, I find a dramatic reduction of kinase activity in *twine*^{1} oocytes. Paradoxically, I detect relatively normal *in vitro* histone H1 Cdk1 kinase activity in *endos*^{00003} mutant stage 14 oocytes, despite the fact that in these mutants Twine and Polo are reduced, and MPM2 epitopes are virtually absent. It is important to point out, however, that although meiotic maturation is significantly delayed in *endos*^{00003} mutants, the vast majority of the oocytes have disassembled their nuclear envelope by stage 14, the stage I used to perform the *in vitro* kinase assay. It is therefore conceivable that *endos*^{00003} mutants eventually reach a threshold of Cdk1/Cyclin B activity that is sufficient to promote nuclear envelope breakdown and may also be sufficient to induce robust *in vitro* phosphorylation of histone H1. It is possible that the low remaining Twine activity (Twine expression at stage 14 is reduced, but not absent) is sufficient to dephosphorylate and activate Cdk1/Cyclin B. Alternative hypotheses, however, could also explain my results. First, it is conceivable that key endogenous substrates (other than histone H1), which are required during meiotic maturation and progression into metaphase I, are not phosphorylated and activated by Cdk1 in *endos*^{00003} mutants. In fact, I have shown that levels of MPM2 phosphoepitopes are drastically reduced in *endos*^{00003} mutants. Most importantly, I have demonstrated, for the first time in *Drosophila*, that these conserved epitopes are generated not only by Polo kinase activity, as previously reported (Logarinho and Sunkel, 1998), but also by the activity of Cdk1.

Several lines of evidence suggest that MPM2 phosphorylated epitopes play important regulatory roles in the cell cycle. DNA topoisomerase II (involved in chromosome condensation and separation of sister chromatids) (Taagepera et al., 1993), mitogen-activated protein kinase $p42^{mapk}$ (involved in inhibition of Cdk1/Cyclin B activity in vertebrates) (Taagepera et al., 1994), *Xenopus* Cdc25 (Kuang et al., 1994), Xenopus Wee1-like kinase (Mueller et al., 1995a) and Microtubule-Associated Proteins MAP1 (Vandre et al., 1986) and MAP4 (Vandre et al., 1991) are examples of proteins that have been identified to be recognized by MPM2 antibodies. I could predict that both Polo and Cdk1 contribute to the phosphorylation of these proteins, independently of each other, or that they promote phosphorylation of different subsets of epitopes. In either case, I would expect to find some MPM2 signal, generated by Polo, in *cdc2* mutants. However, as demonstrated in Chapter II, I find that this is not the case. These results suggest that the generation of MPM2 phosphoepitopes in *Drosophila* depends on the cooperation, likely through a positive feedback loop, between Polo and Cdk1. My results are also consistent with the hypothesis that many of $endos^{00003}$ meiotic defects are caused by the lack of Polo activity.

Cdk1 levels and mobility are affected in $endos^{00003}$ mutants

In addition to finding a reduction in Cdk levels, I observe that the relative abundance of different mobility forms of Cdk1 (likely caused by differences in phosphorylation status) in $endos^{00003}$ mutant oocytes is different from that in $twine^{1}$ or control oocytes based on Western blot analysis using anti-PSTAIR antibodies. The anti-PSTAIR antibody was generated against the conserved (EGVPSTAIREISLLKE) Cdk1

motif and can recognize three differently migrating bands on SDS-PAGE. Previous studies suggested that the upper band corresponds to Cdk1 phosphorylation on both Tyr15 and Thr14 residues, whereas the middle band corresponds to Tyr15 or Thr14 phosphorylation, and the lower band corresponds to unphosphorylated Cdk1 (Choi et al., 1991). It is unclear, however, how phosphorylation of Thr161 factors into the migration of Cdk1 reflected by these bands. Consistent with the requirement for Twine's phosphatase activity to remove both Tyr15 and Thr14 inhibitory phosphates from Cdk1, *twine*^{1} oocytes display hyperphosphorylated Cdk1. However, Cdk1 seems to be hypophosphorylated in *endos*^{00003} relative to control stage 14 oocytes.

Much less attention has been given to the role of activating phosphorylation on the activity of Cdks, mainly because the activity of Cdk-Activating Kinase (CAK), the responsible kinase, is constant throughout the cell cycle (Solomon et al., 1992). Nonetheless, crystal structures studies with Cdk2/Cyclin A have shown that the activating phosphorylation of Thr161 residue is required for the stabilization of the Cdk activating T-loop and thus for its catalytic activity and substrate binding (Kaldis, 1999; Russo et al., 1996). Furthermore, studies of *Drosophila* Cdk7 (the CAK homolog) have shown that Cdk7 is required *in vivo* for Cdk1/Cyclin B activity; the *cdk7* mutant phenotype closely resembles the mitotic phenotype of *cdc2* mutants (Larochelle et al., 1998). Moreover, the levels of Cdk Thr161 phosphorylation oscillate during the *Drosophila* late preblastoderm embryonic cell cycles, suggesting that some level of regulation might exist (Edgar et al., 1994). Therefore, another formal possibility is that in *endos*^{00003} oocytes, Cdk1 is also lacking activating phosphorylation by CAK. Mapping the Cdk1 phosphorylation sites in *endos* mutants relative to controls will be important to begin testing this hypothesis. In

addition, I could also examine phosphorylation of Cdk1 Thr161 in *endos*^{00003} mutants using available phospho-specific antibodies (De Smedt et al., 2002).

Cdk1 associates with Cyclin B in *Drosophila* stage 14 oocytes

I have also shown that Cdk1 and Cyclin B form a complex in stage 14 oocytes. It is known that activation of the Cdk1/Cyclin B complex begins in the cytoplasm, although its nuclear translocation is required to stimulate nuclear events such as nuclear envelope breakdown (Takizawa and Morgan, 2000). Interestingly, phosphorylation of Cyclin B by Cdk1 and Polo has been implicated in promoting Cdk1/Cyclin B nuclear import (reviewed in(Takizawa and Morgan, 2000). Furthermore, localized destruction of Cyclin B in meiotic and mitotic spindles has been reported in *Drosophila* (Edgar et al., 1994; Huang and Raff, 1999; Raff et al., 2002; Su et al., 1998; Swan and Schupbach, 2007). Thus, changes in the Cdk1/Cyclin B complex subcellular localization or local regulation of Cyclin B could also account for a defective Cdk1 activity, as measured by MPM2 levels, in *endos*^{00003} females despite normal *in vitro* H1 Cdk1 kinase activity.

endos, Mtrm, and elgi control the timing of meiotic maturation

The data obtained from my *endos* and *Mtrm* dominant genetic interactions analysis have not only provided new insights for the mechanism of action of *endos* but also uncovered new potential roles for *endos* in meiosis. First, heterozygosity of *endos*^{00003} rescues the precocious breakdown of the nuclear envelope in *Mtrm*$^{\Delta 126}$ heterozygotes. These results are similar to those reported for *polo* heterozygosity (Xiang et al., 2007), based on which it has been proposed that reduction of *polo* compensates for

the decrease in the amount of Mtrm protein available to keep Polo off until meiotic maturation. Similarly, my data suggest that reduction in *endos* dosage decreases Polo levels and thus restores the balance between Polo and Mtrm and the timing of nuclear envelope breakdown.

Although the mechanism of the interaction between Elgi and Endos is less clear, their interaction has not only been demonstrated *in vitro* by my DIVEC biochemical approach, but it has also been confirmed *in vivo* based on immunoprecipitation and mass spectrometry analysis (David Glover, personal communication). Thus, *elgi* most likely plays a role in the *endos* pathway. Based on the similar premature presence of metaphase I DNA structures that I observe between $elgi^{1}$ mutants and $Mtrm^{\Delta 126}$/+ females, it is possible that Endos suppresses Mtrm activity by inhibiting Elgi. Although the mammalian Elgi homolog, Nrdp1, functions as an E3 ubiquitin ligase to promote the degradation of two different proteins (i.e. Erb3 and Erb4 receptor tyrosine kinases and inhibitor-of-apoptosis protein BRUCE) (Qiu and Goldberg, 2002; Qiu et al., 2004), additional targets must exist in *Drosophila* (fly genome lacks Erb proteins). For example, Elgi might be modifying the activity of Mtrm. Monoubiquitination or polyubiquitination of lysine residues other than Lys48 does not normally result in protein degradation. Instead some of these post-translational modifications have been shown to modulate the localization, activity, trafficking or endosomal sorting of target proteins (Johnson, 2002; Schnell and Hicke, 2003). Therefore, a completely hypothetical scenario is that the posttranslational modification induced by Elgi could potentially be promoting the interaction between Mtrm and Polo prior to the onset of oocyte meiotic maturation. In any case, to better understand the mechanism by which Endos and Elgi regulate the

meiotic cell cycle, it will be important to determine whether Elgi acts as an E3 ubiquitin ligase in flies and to identify the Elgi targets (see Future Directions).

Mtrm is not degraded prior to meiotic maturation but may instead be regulated by posttranslational modification

My Mtrm expression data differs from the current model that proposes that Mtrm degradation at stage 13 releases Polo inhibition and promotes meiotic maturation (Xiang et al., 2007). Instead, I find that Mtrm expression peaks at stage 14 (Figure 3.3, Chapter III). Thus, my data is consistent with an alternative model that predicts that inhibition of Mtrm activity, rather than its degradation, is the trigger for meiotic maturation. Indeed, I observe a shift in Mtrm mobility in stage 14 oocytes, and in *endos* mutants both the levels and electrophoretic mobility of Mtrm are altered. Based on these observations I hypothesize that *endos* might be regulating Mtrm's posttranslational modification at this developmental stage (see below). I could then predict that while Mtrm inhibits Polo activity until the onset of nuclear envelope breakdown, *endos* not only is required for Polo stability, but it may also be required to overcome the Mtrm inhibition of Polo, which is required to activate Twine and initiate the cascade of events that culminate in the disassembly of the nuclear envelope.

endos, Mtrm, and gwl may regulate heterochromatin pairing

The *Drosophila* fourth chromosomes use heterochromatin pairing, rather than genetic exchange (chiasmata), as a mechanism to link homologs and to orient their centromeres toward opposite poles during prometaphase (Dernburg et al., 1996; Hawley et al., 1992; Karpen et al., 1996). This centromeric orientation ensures migration of the

fourth chromosomes to opposite poles during metaphase I, and eventually, their proper segregation during anaphase I (Hawley et al., 1992; Hawley et al., 1993). Although it was not clear whether this phenotype is a secondary consequence of the precocious nuclear envelope breakdown, $Mtrm^{\Delta 126}$ heterozygotes also fail to properly segregate their fourth chromosomes to opposite poles; hence their high frequency of achiasmate non-disjunction (Xiang et al., 2007). Interestingly, this defect can also be fully suppressed by reducing the dosage of *polo*, thereby suggesting that high Polo levels impair heterochromatin pairing and could lead to non-disjunction defects. This is not surprising, considering the known role of Polo in the negative regulation of sister chromatid cohesion (Alexandru et al., 2001; Clarke et al., 2005) and the fact that cohesins may be involved in heterochromatin pairing (McKee, 2004).

Recently, it has been shown that the Greatwall (Gwl) kinase antagonizes Polo activity during meiosis (Archambault et al., 2007). Specifically, these studies have shown that homozygosity of the *Scott of the Antartic* (*Scant*) mutation in the *gwl* gene, which generates a hyperactive Gwl kinase, can rescue the non-disjunction defects caused by *Mtrm* haplo-insufficiency (Archambault et al., 2007). A loss-of-function mutation in the *gwl* splice acceptor site, gwl^{Sr18}, causes stage 14 yolk distribution defects, failure of progression into metaphase I due to scattered chromosome masses, and sterility (Archambault et al., 2007). These phenotypes are similar to the ones I observe in *endos* and *twe* mutants. Interestingly, these studies also reported that the dispersed chromosome masses observed in gwl^{Sr18} mutant oocytes are due to defects in sister chromatid cohesion.

Contrary to the effect observed on the timing of meiotic maturation, reduction of *endos*^{00003} dosage also enhances the fourth achiasmate chromosome misorientation defects of *Mtrm*$^{\Delta 126}$/+ heterozygotes. These findings are consistent with a role of *endos* in promoting heterochromatin pairing. It will be interesting to examine genetic interactions between *endos* and *gwl* (see Future Directions) and to further characterize in more details this chromosome alignment (orientation) defect in different mutant combinations. In addition to identifying a potential and unanticipated role of *endos* in heterochromatin pairing, my *endos-Mtrm* genetic interactions results clearly demonstrate that defects in heterochromatin pairing of Mtrm heterozygotes are not a secondary consequence of defects in the timing of meiotic maturation.

endos regulates Mtrm levels potentially via a posttranslational modification

Considering that Mtrm is phosphorylated at six different residues (T40, S48, S52, S121, S123, S124) (Xiang et al., 2007), it is plausible that changes in phosphorylation may dictate its stability and/or activity, and that this posttranslational control may depend on *endos'* function. These hypotheses are supported by the observations that Mtrm mobility is affected in *endos*^{00003} stage 14 oocytes (only a presumed phosphorylated form of Mtrm is present) and that Mtrm levels at stages 13 and 14 are drastically reduced in *endos*^{00003} mutants. I should also point out that *twine*^{1} stage 14 oocytes also display strong reduction of Mtrm (see Chapter III). Therefore, it is possible that the regulation of Mtrm phosphorylation at stage 14 might be involved in Mtrm stability. I could speculate that *endos* might be contributing to Mtrm stability by regulating directly or indirectly Twine or Cdk1 levels and/or activities. Although the mechanism is currently unknown, *gwl* and

endos might also work in a similar pathway. Studies with *Xenopus* oocytes have shown that Gwl promotes Cdc25 phosphorylation and activation (Zhao et al., 2008). Therefore, it is also possible that *endos* might be involved in the activation of Gwl, and that this activation indirectly leads to dephosphorylation of Mtrm. One way I could begin testing these models is by analyzing Mtrm mobility in *gwl* loss-of-function mutants as well as in *gwl,endos* and *scant,endos* double mutants. One prediction from this model is that I will only detect a phosphorylated form of Mtrm in *gwl* or *gwl,endos* double mutants. In contrast, in *scant,endos* double mutants, where Gwl is hyperactive, I should detect a dephosphorylated form of Mtrm. These results would be consistent with the role of a phosphatase in mediating the stability of Mtrm under the control of Endos and/or Gwl.

Endos might be regulating the activity of the APC/C

The levels of four key cell cycle regulators including Twine, Polo, Cdk1, and Mtrm are affected by *endos* loss-of-function. This is not a non-specific effect, however, because other proteins such as Cyclin B are expressed normally in *endos* mutants. Given that Polo, Twine and Cdk1 are APC/C targets upon M-phase exit (Businо et al., 2004; Donzelli et al., 2002; Eckerdt and Strebhardt, 2006; Vodermaier, 2004), it is possible that *endos* regulates the APC/C activity itself. Excessive or slightly premature APC/C activation in $endos^{00003}$ mutants could explain the reduction in protein levels that we observe. I could address this possibility by reducing the activity of APC/C and testing for rescue of the meiotic defects in $endos^{00003}$ mutants (see Future Directions).

Endos phosphorylation is dispensable for its meiotic roles whereas dephosphorylation appears to be required fourth chromosome proper alignment

Taking into consideration that the α-endosulfine predicted PKA phosphorylation site is conserved across species, it is surprising to see rescue of all *endos'* meiotic defects and sterility by the expression of the Endos^{S107A} non-phosphorylatable mutation. However, I do find that a phosphomimetic mutation at this site impairs *endos'* potential role in heterochromatin pairing. Germline expression of the phosphomimetic Endos^{S107D} mutation in the background of *endos*^{00003} mutants resembles the achiasmate chromosome misorientation defects of *Mtrm*$^{\Delta 126}$/+ females. It is tempting to speculate that this mutation mimics phosphorylation of S107 and that dephosphorylation of Endos is required for fourth chromosome proper alignment via any of a number of possible mechanisms. I cannot rule out the possibility, however, that Endos^{S107D} is not mimicking phosphorylation of Endos and, instead, is simply a defective protein that is incompletely rescuing the *endos* meiotic defects. As I discussed in Chapter III, testing whether Mtrm and Polo levels are affected by this phosphomimetic mutation will help clarify how this site may be required to control Endos.

endos is maternally required for early embryonic mitoses

Several lines of evidence point to *endos* as an important player in the mitotic cell cycle. First, a small fraction of *endos*^{00003}-derived mutant embryos seem to initiate embryonic mitoses but do so aberrantly. Second, *Mtrm*$^{\Delta 126}$ +/+ *endos*^{00003} double heterozygotes and *endos*^{S107D}-expressing females show severe reduction in fertility regardless of the normal metaphase I DNA structures that I detect in a significant fraction of stage 14 oocytes. These findings are consistent with a role of maternal *endos* during

the embryonic syncytial mitoses. In support of my conclusions, more direct roles of *endos* in mitosis have been identified. In a large-scale screen for genes that are required for mitotic spindle assembly in *Drosophila* S2 cells, *endos* was identified as being required for proper chromosome alignment and spindle formation (Goshima et al., 2007). A more detailed characterization of the *endos'* mitotic defects will help further clarify the function of *endos* during the early mitotic cell cycle and compare it to its meiotic role.

Model for the roles of *endos* during meiosis

Based on my *endos* mutant analysis, genetic interaction and regulation data described in Chapters II and III, I have arrived at a general working model to explain how *endos* might be regulating two separate processes during meiosis in *Drosophila*, namely meiotic maturation and chromosome orientation/alignment during metaphase I (Figure 5.1). According to this model, Endos initially positively regulates Polo levels in part to activate Twine (Figure 5.1A). Activated Twine removes inhibitory phosphates from Cdk1/Cyclin B to trigger the initiation of events that lead to meiotic maturation, including nuclear envelope breakdown, chromosome condensation and bipolar spindle formation. In addition, Endos binding to Elgi is required to inhibit Elgi's activity and refine the timing of Cdk1/Cyclin B activation, potentially via the modulation of the Mtrm-Polo interaction. Once meiotic maturation has taken place, Endos may be required to control the phosphorylation status and stability of Mtrm, ultimately to control heterochromatin pairing (Figure 5.1B). Alternatively, Endos may regulate heterochromatin pairing by inhibiting Polo directly or via activation of Gwl. Finally, Endos dephosphorylation may be required for the pathway that leads to heterochromatin pairing, achiasmate fourth

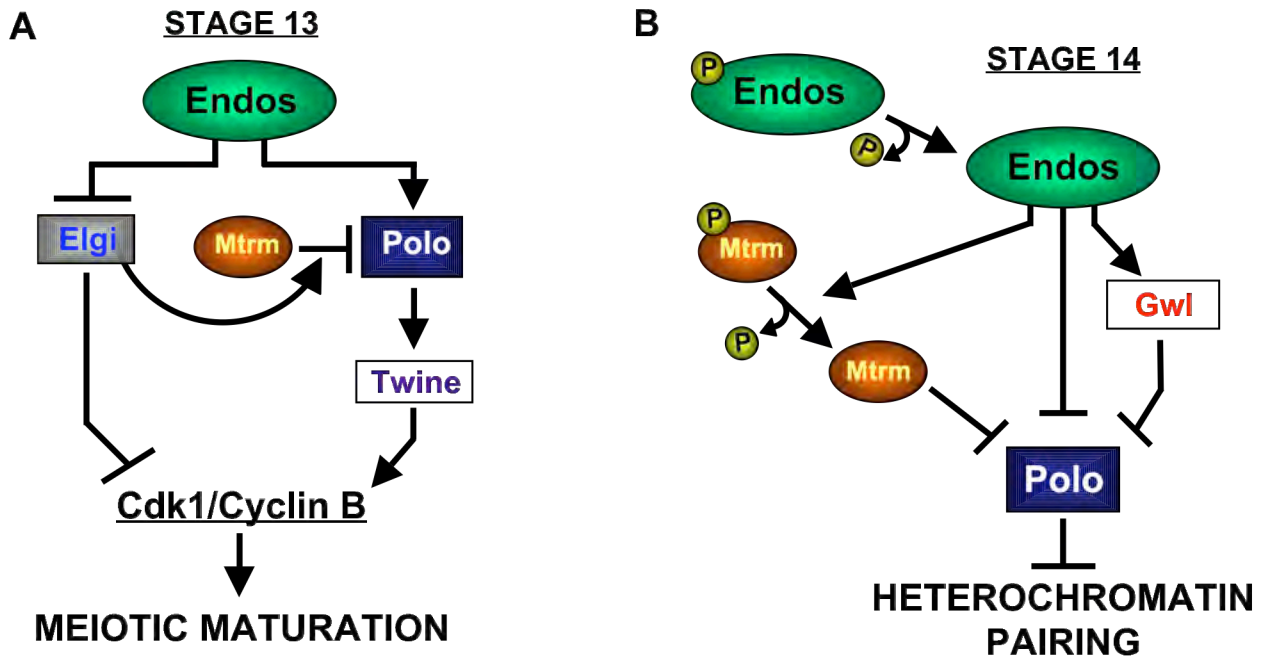

Figure 5.1. Models for the role of *endos* in regulating the timing of meiotic maturation and heterochromatin pairing. (A) We propose that Endos positively regulates the timing of meiotic maturation by regulating the levels of Polo, which is required to induce activation of Twine and Cdk1/Cyclin B and promote meiotic maturation. In addition, Elgi inhibition by Endos alleviates the inhibition of Polo by Mtrm to refine the timing of meiotic maturation. (B) We hypothesize that after meiotic maturation, at stage 14, Endos inhibits Polo activity directly to promote heterochromatin pairing and achiasmate fourth chromosome proper orientation and segregation at anaphase I. It is also possible that Endos plays a role in this process by regulating Mtrm protein levels or its stability by promoting its dephosphorylation. Finally, dephosphorylation of Endos might be required to regulate additional functions in heterochromatin pairing, perhaps via interactions with Gwl.

chromosome orientation to opposite poles and proper achiasmate chromosome segregation during meiosis I.

Endos meiotic functions may be conserved through evolution

My studies demonstrate a previously unknown central role for Endos in oocyte meiotic maturation and, perhaps, a role during the heterochromatin pairing process. Considering the high conservation at the amino acid level between α-Endosulfines across vertebrate and invertebrate species it is very likely that the molecular function of α-Endosulfine has been conserved through evolution. Consistent with this prediction, the human protein can significantly rescue all of the meiotic defects of *endos*^{00003} mutants. In addition, the strong α-Endosulfine expression detected in mammalian oocytes is consistent with the hypothesis that, in mammals, meiotic maturation may also require the activity of *endos.* Based on this hypothesis I could also predict that mammalian oocytes may require the activity of α-Endosulfine to undergo meiotic maturation. It will be very interesting to test the role of *α-endosulfine* in *Xenopus* oocytes and also to generate a germline-specific *α-endosulfine* knockout mouse in the future to analyze a potential role of this gene in meiosis.

Understanding the role of α-Endosulfine could potentially provide new insights into the causes of infertility or aneuploidy in humans. As discussed in Chapter I, the tight coordination between meiosis and gametogenesis is critical to avoid chromosomal abnormalities. Aneuploidy, for example, arises as a result of defects in oocyte arrest or meiotic maturation. The meiotic defects of *endos*^{00003} mutants, for example, are the only cause of their sterility. Interestingly, a connection between human aneuploidy and

heterochromatin pairing has also been proposed (Koehler and Hassold, 1998). In humans, abnormalities such as trisomy 21, trisomy 18, trisomy 15, and sex chromosome trisomy are likely caused by achiasmate chromosome non-disjunction (Koehler and Hassold, 1998). Moreover, it has been proposed that a similar "backup" segregation mechanism of achiasmate chromosomes might also function in mammalian meiosis (Koehler and Hassold, 1998). Therefore, the role of *endos* in ensuring heterochromatin pairing in *Drosophila* serves as a model to understand this phenomenon in humans.

Future Directions

It is clear that the intricate connections between *endos*, *polo*, *twe*, *Mtrm*, and *gwl* need to be explored further. Understanding how these genes act with respect to each other will help me clarify the models described above. To accomplish this goal, I plan to perform additional dominant or recessive genetic interaction tests between these genes. The outcome of these experiments will provide key clues as to where to place these genes relative to each other in the *endos* pathway. In addition, we will determine how Polo, Twine, and Mtrm levels of expression and mobility change in these different genetic backgrounds and whether they are altered by the above genetic interactions. These results will allow me to test many aspects of my current working models.

To date, Elgi is the only true direct physical interactor identified for Endos, and this interaction has been shown both *in vitro*, by me, and *in vivo* (David Glover, personal communication). To better understand the role of Elgi in the Endos pathway I will need to test whether Elgi has *in vitro* ubiquitin ligase activity and whether this activity is required *in vivo*. Given that almost all known Really Interesting Novel Gene (RING)

finger E3 ubiquitin ligases are capable of mediating their own ubiquitination, a sensitive indicator of *in vitro* E3 ligase activity, I will combine recombinant E1 and E2 enzymes with Elgi and look for auto-ubiquitination. A preliminary attempt (Former Research Assistant, Li Zhang) suggested that this will be feasible. It will also be crucial to identify the substrates of Elgi. One potential approach to identify these targets could be to perform *in vitro* ubiquitination assays using the DIVEC approach. In addition to the first *Drosophila* Gene Collection (DGC) of full-length cDNAs, which I used in our DIVEC screen, we have now available to the fly community three more collections that altogether cover most, if not all, of the genome. The identity of these targets and elucidation of their roles *in vivo* will shed light on how Elgi regulates meiosis in *Drosophila* females.

Although my evidence suggests that protein instability is not a general defect in *endos*^{00003} mutants (based on unaltered levels of Cyclin B or actin), I do find that levels of key cell cycle proteins are drastically reduced in these mutants. Moreover, although I can detect overexpressed Twine and Polo in control oocytes, I am not able to drive significant levels of expression of these proteins in *endos* mutants. One potential explanation is that an abnormally hyperactive APC/C complex is targeting these proteins for degradation. Mutations disrupting many *Drosophila* APC/C subunits including *morula/APC2* or *Cdc16/APC6*, or APC/C activators such as *cortex*, are currently available (Kashevsky et al., 2002; Pal et al., 2007; Pesin and Orr-Weaver, 2007; Reed and Orr-Weaver, 1997; Swan and Schupbach, 2007). Therefore, to begin investigating this possibility, I will decrease the activity of the APC/C by decreasing or eliminating the function of these subunits or activators in *endos* mutants and asking whether I can partially or completely rescue any of their meiotic defects.

My findings clearly demonstrate that *endos* is involved in regulating multiple steps of female meiosis. Thus, identifying new *endos* partners that could act as inhibitors or regulators of its activity will ultimately help us elucidate in more detail the mechanisms triggering meiotic maturation in *Drosophila*. To identify these genes, our lab plans to employ different approaches: **(1)** Based on my previous observations, I have proposed to use the oocyte dehydration phenotype as a potential hallmark for genes required for meiotic maturation. We will screen the female sterile Zuker collection (Koundakjian et al., 2004; Wakimoto et al., 2004), which encompass EMS-induced mutations in approximately 12,000 genes on the II and III chromosomes, for ovaries containing non-dehydrated oocytes (a defect that is shared by *endos, cdc2, twine* and *Mtrm* mutants). Candidate mutant lines will subsequently be tested for specific defects in meiotic maturation or chromosome orientation in stage 14. Our Research Assistant Kimi LaFever has begun screening this collection and has so far identified 8 potential candidates that partially fail to dehydrate their stage 14 oocytes, out of 593 screened lines. In the near future, our laboratory will perform complementation tests with *endos, twe* and *Mtrm* to determine whether these candidates are new alleles of these genes or novel regulators. **(2)** We will also screen the *Drosophila* deficiency (Df) kit, which contains approximately 300 deficiencies, for dominant genetic interactions with *endos* or *Mtrm*. We find that $Mtrm^{\Delta 126}$ +/+ $endos^{00003}$ double heterozygous flies are almost completely sterile. Thus, in this deficiency screen, we will be looking for similar dominant genetic interactions and testing for strong sterility in *Df/+;* $endos^{00003}$/+ or *Df/+;* $Mtrm^{\Delta 126}$ /+ females. **(3)** The last approach consists of screening the Bruinfly collection (Chen et al., 2005), which contains approximately 900 FRT-lethal lines, by

generating germline mutant clones and looking for defects in meiotic maturation in DAPI-stained oocytes, as described in Chapters II and III. Screening the Bruinfly collection will allow us to identify genes with potential roles in meiosis irrespective of their essential roles during development. This screen will be conducted by Min-Young Kim (Graduate student). The results obtained from these screens will be very valuable to identify not only new components in the *endos* pathway but also novel meiotic maturation regulators.

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