The Oocyte-to-Embryo Transition: Regulation of Oocyte Maturation and Egg Activation in Drosophila

by

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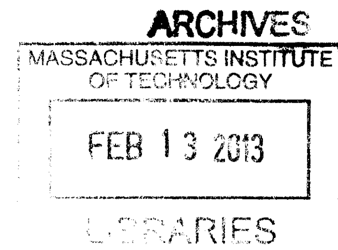

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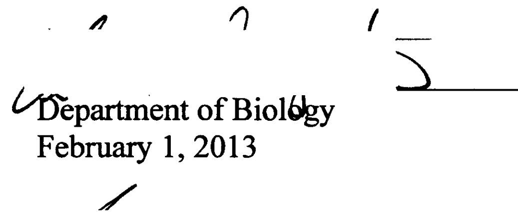

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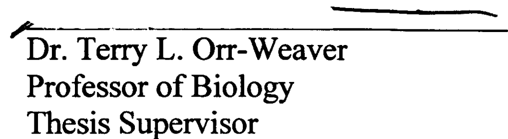

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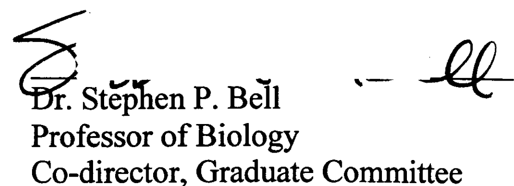

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Abstract

In oogenesis, meiosis must be highly regulated to ensure that growth of the oocyte and chromosomal segregation are coordinated properly. To do this, meiosis arrests at two points to permit oocyte differentiation and coordination with fertilization. In Drosophila, the first arrest in prophase I is released by oocyte maturation, and the second arrest in metaphase I is released by egg activation. This thesis explores mechanisms controlling these two processes. First, the putative role of the Deadhead (DHD) thioredoxin in Drosophila female meiosis is examined. Possible roles that DHD may play in DNA replication, ROS/RNS redox pathways, and vitelline membrane crosslinking are explored. Furthermore, current research into the role of Ca^{2+} as a regulator of Drosophila egg activation is summarized. Recent studies have suggested that Sarah (Sra), a regulator of Calcineurin (CN), is required for egg activation and meiotic completion. A model for Sra/CN signaling is presented, highlighting the role of Ca^{2+} in Drosophila activation, and emphasizing aspects of meiotic activation conserved across species. Finally, proteins recovered from a large-scale proteomic screen undertaken by our lab are discussed. This screen identified proteins that increase or decrease significantly during the processes of maturation and activation through quantitative mass spectrometry. Pairwise comparison of protein levels between pre- and post- maturation oocytes (stage 10 vs. stage 14 oocytes) or pre- and post-activation eggs (stage 14 vs. unfertilized eggs) identified candidate proteins up- and downregulated during one or both of these processes. These candidates include proteins involved in calcium binding and transport, the ubiquitination pathway, steroid biosynthesis and metabolism, and a gap junction protein. Additional characterization of these proteins may provide further insight into the regulation of Drosophila maturation and activation.

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INTRODUCTION

Meiosis

While most cells in the body undergo the cell division process of mitosis, in which one cell divides into two daughter cells, each with the same number of chromosomes as the parent cell, the germ cells undergo a specialized type of division. Oocytes and spermatocytes undergo the process of meiosis. In meiosis, two rounds of chromosome segregation occur without an interrupting round of DNA replication, producing haploid sperm cells or a haploid oocyte (and 3 unused polar bodies) depending on the gonadal sex. This specialized cell division is necessary to ensure the zygote has the proper number of chromosomes after the egg and sperm fuse at fertilization.

The sperm and egg cells contribute different things to the embryo: the sperm primarily contributes genetic information, but the egg contributes both genetic information and components of its cytoplasm, including stockpiled mRNA. Since the first mitotic divisions of the embryo are under maternal rather than zygotic control, this maternal mRNA created during oogenesis is necessary to control embryonic cell processes, including mitosis and embryo patterning, prior to the transition to zygotic control. In Drosophila, approximately the first 13 mitotic divisions during embryogenesis occur under maternal control, and key aspects of mitosis are regulated translationally, rather than transcriptionally (Vardy and Orr-Weaver, 2007).

To permit oocyte differentiation and the stockpiling of maternal components, it is necessary to have arrest points during the meiotic divisions. This allows for the growth of the oocyte and follicle cells (somatic cells surrounding the oocyte) to be coordinated with chromosome separation to ensure the egg develops properly. There are two arrest points in most species, other than *C. elegans*, in which oocytes only arrest once. The first arrest point occurs in prophase I in all organisms, and the process of oocyte maturation releases this arrest. The second

arrest point occurs at metaphase II in most vertebrates, including mammals, and is released through the process of egg activation that occurs upon fertilization of the oocyte. In Drosophila and other insects, however, the secondary meiotic arrest occurs in metaphase I and egg activation is fertilization independent (Von Stetina and Orr-Weaver, 2011). Instead, activation appears to be induced upon the passage of the oocyte through the oviduct and uterus of females, where mechanical pressure and osmotic pressure (from rehydration) initiate activation (Mahowald et al., 1983; Page and Orr-Weaver, 1997; Horner and Wolfner, 2008). In addition to meiotic resumption, activation in Drosophila also results in crosslinking of the vitelline membrane and increased protein translation (Von Stetina and Orr-Weaver, 2011). The mechanisms controlling oocyte maturation and activation in Drosophila will be the focus of this thesis.

Control of Meiosis in Drosophila

Control of meiosis and oocyte development in Drosophila has been a topic of intense research over the past few decades. Due to the ease with which this organism is grown and manipulated, and the thorough characterization of its meiotic stages, it is one of the model organisms to study this process. While the upstream signals that initiate maturation are unknown, much is understood about how prophase I arrest is maintained and how this arrest is released.

To fully understand meiosis in Drosophila, a basic understanding of the structure of the ovary is needed. Initially, a single germ line stem cell divides to give rise to the cystoblast, which undergoes 4 incomplete mitotic divisions, forming 16 individual cells connected by cytoplasmic bridges. Only one of these 16 cells becomes the egg cell, while the others become the nurse cells that provide the egg with nutrients, mRNA, and other molecules required by the embryo during the maternally-controlled mitotic divisions in early embryogenesis. The egg chambers (oocyte,

nurse cells, and follicle cells) are designated a stage, from Stage 1 to Stage 14, as development proceeds. Meiosis begins in the germarium, with chromosomes compacting into a visible structure called a karyosome at the end of prophase I, and the oocyte remains in this state until the (unknown) signal for maturation is received (**Figure 1.A**). While the chromosomes remain arrested, the rest of oocyte development proceeds (Spradling, 1993)

The major players controlling the primary arrest in prophase I and maturation are shown in **Figure 2**. Matrimony binds to and inhibits Polo kinase stoichiometrically, which keeps Cyclin B/Cdk1 from activating. Upon maturation, levels of Polo rise, until the excess of Polo is able to activate the phosphatase Twine/Cdc25 through phosphorylation, which then activates Cdk1 through dephosphorylation (Xiang et al., 2007; Von Stetina et al., 2008). α-Endosulfine (Endos) is an upstream regulator of maturation and works in multiple ways; it enhances the stability of Polo and Twine/Cdc25, and it interacts with and inhibits Early girl (Egli), an E3 ubiquitin ligase (Von Stetina et al., 2008)

The process of activation in Drosophila is much less well understood. Arrest at metaphase I in the oocyte is due primarily due to recombination between homologous chromosomes producing physical attachments called chiasmata (Jang et al., 1995). Interaction between heterochromatic regions not undergoing recombination may also play a role in maintaining the attachment (Hawley et al., 1992). Cyclin B/Cdk1 also is involved in the metaphase I arrest, and Cyclin B must be degraded for meiosis to progress (Swan and Schupbach, 2007). The Anaphase Promoting Complex / Cyclosome (APC/C) is an E3 ubiquitin ligase that regulates the progression of meiosis and mitosis. It targets various substrates for degradation through ubiquitination at specific sites (D-box, KEN box, A-box and O-box) (Acquaviva and Pines, 2006). The APC/C is activated by Fizzy/Cdc20 (FZY) and the female-meiosis specific

activator Cortex (CORT) in meiosis I and CORT in meiosis II (Page and Orr-Weaver, 1996; Pesin and Orr-Weaver, 2007, 2008). Pressure exerted on the egg as it passes through the oviduct and uterus causes activation (Mahowald et al., 1983; Horner and Wolfner, 2008), but other signals and the internal mechanisms of signal transduction in the egg are still unclear. Research has suggested Ca^{2+} pathways are involved in activation, through action of Sarah (Sra) and Calcineurin (see section II).

Meiosis in mammals: connections and commonalities

In mammals, as in flies, prophase I arrest is maintained by preventing the activation of Cyclin B/Cdk1 (Sagata, 1996). This maintenance involves high cAMP levels and APC/C mediated degradation of Cyclin B, both of which limit Cyclin B/Cdk1 activity prior to maturation (Reis et al., 2006; Vaccari et al., 2008; Norris et al., 2009; Schindler and Schultz, 2009). Unlike in Drosophila, where no initiation signal(s) for maturation has/have been identified, mammals use luteinizing hormone (LH) to initiate meiotic resumption from prophase I (Neal and Baker, 1975). LH inhibits both cGMP production and cGMP import into the oocyte through gap junctions, which decreases cAMP levels and activates Cyclin B/Cdk1 (Sela-Abramovich et al., 2005; Norris et al., 2008, 2009) .Therefore, in mammals, the interaction between the oocyte and surrounding somatic cells through gap junctions is important for maintaining and ending the primary arrest. Follicle cells have not been shown to interact with oocytes to control meiosis in Drosophila, but Von Stetina and Orr-Weaver suggest that communication through gap junctions between follicle cells and the oocyte may play a role in meiotic regulation (2011). Experiments show the gap junction proteins called Innexins are expressed during oogenesis in the nurse cells, oocyte, and follicle cells, and that antisera against Innexin-2 (Inx2) (a component of gap junctions in Drosophila) limits oocyte growth, follicle cell development, and eggshell formation

(Stebbings et al., 2002; Bohrmann and Zimmermann, 2008). The identification of Innexin 4 (Inx4) as a protein that decreases significantly during activation (Kronja and Orr-Weaver, unpublished) lends support to the hypothesis that gap junction proteins may mediate communication during oogenesis (see section III).

After maturation, chromosomes arrest again at metaphase II, and this arrest is maintained by the Emi2 pathway and the MOS/MAPK pathways through inhibition of the APC/C, and consequentially, stabilization of Cyclin B/Cdk1(Araki et al., 1996; Kalab et al., 1996; Madgwick et al., 2006; Shoji et al., 2006). The cohesin rings encircling the chromosomes and keeping sister chromatids together are maintained as long as the protein Securin is phosphorylated and stabilized, which sequesters and inactivates its binding partner Separase (Yanagida, 2005). After fertilization, waves of Ca^{2+} throughout the oocyte induce activation through Calmodulin-Dependent Protein Kinase II (CaMKII), which leads to downstream APC/C activation (Tatone et al., 2002; Jones, 2005; Backs et al., 2010). Dephosphorylation of Securin leads to its ubiquitination by the APC/C and subsequent proteasome degradation. This, in turn, activates Separase, which cleaves the cohesin rings and allows for chromosome separation. Activating the APC/C also leads to Cyclin B degradation, which is crucial for meiosis II progression (Jones, 2005). A regulatory role for calcium is emerging in Drosophila activation, emphasizing the conservation of meiotic processes between insects and vertebrates (see section II).

I. Redox and meiosis: The Deadhead thioredoxin (DHD) is required for meiosis in Drosophila

Discovery of *deadhead*: role of thioredoxins in meiosis

Thioredoxins are proteins that modulate the reduction of cysteine residues and control disulphide bond formation (Holmgren, 1989; Buchanan et al., 1994). Since many regulatory

proteins, such as phosphatases, kinases, and translational machinery may be activated through redox processes, thioredoxins play an important regulatory role in various systems throughout the cell (Holmgren, 1989). In Drosophila, there are 3 members of the thioredoxin family; 1) Trx-2, a ubiquitous thioredoxin, 2) Deadhead (DHD), an ovary-specific thioredoxin, and 3) TrxT, a testis-specific thioredoxin (Salz et al., 1994; Bauer et al., 2002; Svensson et al., 2003). A role for DHD has been identified in meiosis in *Drosophila melanogaster.* Salz et al. discovered that *dhd* is a female-specific maternal-effect gene that is required for the completion of meiosis in Drosophila (1994). Females homozygous for either a targeted deletion over the *dhd* locus or a *P*-element insertion, both of which disrupt expression of *dhd,* lay eggs that do not complete meiosis. 90% of these eggs show irregular polar body structure, with chromosomes most commonly arresting in anaphase I. The rare eggs with enough DHD function to continue through to the mitotic phase of embryogenesis showed asynchronous mitotic divisions, errors in cell migration to the cortex of embryos, and occasionally differences in the ploidy of nuclei (Salz et al., 1994). This evidence points to a requirement for the DHD thioredoxin in female meiosis.

The molecular function of *dhd* in meiosis, however, has not been elucidated. Over the years, conflicting data have surfaced regarding the stage(s) at which DHD is required. In fact, some studies suggest *dhd* eggs arrest after completion of meiosis, but before embryogenesis (Page and Orr-Weaver, 1996; Elfring et al., 1997). Possible roles for this thioredoxin in meiotic regulation and progression are explored below.

Developmental expression

The expression of the *dhd* gene during Drosophila development and reproduction seems to support a role of this thioredoxin in meiosis, egg development, and/or embryogenesis. After the role of *dhd* in female meiosis was established, the expression of this gene was studied. Salz et

al. showed through Northern Blot that *dhd* mRNA is visible in the ovary starting at stage 9 in oogenesis, and by stage 10B the nurse cells adjacent to the developing oocyte show a high level of *dhd* expression (Salz et al., 1994). Later, protein localization of DHD in Drosophila was assessed using fluorescent imaging of a *dhd-eCFP* construct. In this experiment, the fluorescent fusion protein was visible as early as stage 3 in the nuclei of the oocyte and nurse cells and remained present throughout oogenesis, while it was not present in the follicle cells (Svensson et al., 2003). This construct was not fully functional and was unable to restore DHD function, so it is possible that the localization observed in these experiments is not representative of the endogenous protein.

Data from our lab has demonstrated that the regulation of DHD is highly dependent on the progress of oogenesis. Through a large-scale proteomic study, we found that DHD levels increase significantly between stages 11 and 14 (when maturation occurs) and decrease between stages 14 and the unfertilized egg (when activation occurs) (Kronja and Orr-Weaver, unpublished data). These data suggest highly regulated DHD expression, which is intimately tied to the timing of maturation and activation.

Possible Roles of Deadhead in Drosophila

A conserved role for thioredoxins in DNA replication

Early studies of the role of thioredoxin found that enzymes were required in DNA replication in viruses, and were identified as a part of the DNA polymerase complex in the T7 phage (Mark and Richardson, 1976; Adler and Modrich, 1983; Bedford et al., 1997). The role of thioredoxins in DNA replication seems to be conserved in yeast and Xenopus. In *S. cerevisiae*, there are two thioredoxin genes (*trx1* and *trx*2). *trx1 trx2* double mutants show a much slower S phase in vegetative, dividing cells, which leads to a shorter G1 phase, maintaining a constant

total length of the cell cycle (Muller, 1991, 1995, 1996). Evidence showed that this effect is dependent on the redox activity of thioredoxin, and is due to a reduction in the activity of ribonucleotide reductase, the enzyme that maintains dNTP pools. This decreased activity reduces the levels of dNTPs available for DNA replication in the *trx1 trx2* double mutants, slowing S phase (Koc et al., 2006)

In Xenopus, thioredoxins may also play a role in DNA synthesis, but through a different mechanism. Hartman et al. found that injections of thioredoxin protein from sufficiently divergent species (including spinach thioredoxin m, as well as *E. coli* thioredoxin) inhibit S-phase DNA synthesis in the Xenopus egg when injected shortly after fertilization (1993). Since this effect is not observed with thioredoxin protein purified from species more related to Xenopus, the authors hypothesized that the inhibition is due to the spinach thioredoxin m being able to perform some but not all functions of the endogenous Xenopus thioredoxin due to sequence differences. Measuring the incorporation of radioactively labeled dCTP that is injected into the embryo along with the spinach protein shows that incorporation of nucleotides during DNA replication is severely impaired. Order-of-addition experiments further suggest that the inhibition of endogenous Xenopus thioredoxin impairs the initiation of DNA synthesis, rather than elongation. The possibility that this effect is due to impurities in the protein preparation was dismissed, as repeated purification of the protein using 4 different methods all resulted in the same observations. Reduction of the thioredoxin with NEM (N-ethylmaleimide) to kill redox activity prior to injection did not eliminate its inhibitory effects. Therefore, in this organism, unlike in yeast, at least one role of thioredoxin in DNA replication is not redox-dependent (Hartman et al., 1993).

Pellicena-Palle et al. show, through point mutations of the two conserved cysteines in the active site of the protein, that the function of DHD in the completion of meiosis in Drosophila is dependent on the redox activity of this enzyme (1997). These point mutation sites are analogous to mutations made in human thioredoxin that result in a significant change in the secondary structure of the human protein (Oblong et al., 1994). They show that DNA synthesis proceeds in the absence of DHD activity in *giant nuclei (gnu), plutonium (plu),* and *pan-gu (png)* null embryos (which show the giant nucleus phenotype due to DNA replication in the absence of nuclear division in embryos) (Pellicena-Palle et al., 1997). From this, it was concluded that DHD is not required for DNA synthesis in Drosophila. This experiment, however, does not examine whether DNA synthesis in the *plu, gnu* and *png* null embryos continues at a normal rate. DNA replication may be slowed, as it is in *trx1 trx2* yeast mutants. Also, the ubiquitous thioredoxin, Trx-2, may perform a redundant function in embryonic DNA replication. Finally, precise quantification of DNA levels in wild-type and mutant embryos was not performed, so while DHD may not be necessary for DNA replication, this does not exclude the possibility it is involved in this process. Further investigation is required to determine whether *dhd* eggs show impaired DNA synthesis or regulation of DNA synthesis.

Redox and ROS in oogenesis

It is interesting that Drosophila lacks a glutathione reductase system. Thioredoxins (including DHD) are able to reduce glutathione (GSH), and thioredoxins may replace glutathione reductase, which may pose an interesting possibility for the role of DHD in oogenesis (Kanzok et al., 2001). In mammals, it has been shown that redox processes (especially the reduction and oxidation of glutathione) are tied to stages of egg development. GSH is oxidized through reactions with reactive oxygen species (ROS) (see **Figure 3.A**), and it is one of the main

regulators of ROS in cells. Reactive oxygen species (such as H_2O_2) appear to be important for regulating maturation in mice, with different effects based on the concentrations of H_2O_2 in the oocyte. Prophase I arrested mouse oocytes exposed to high H_2O_2 concentrations *in vitro* are unable to undergo maturation; visualized by inhibition of both germinal vesicle breakdown and first polar body extrusion (Chaube et al., 2005, 2008, 2009; Tripathi et al., 2009). However, the addition of low levels of H_2O_2 to rat oocytes *in vitro* induces maturation, suggesting a range of concentrations in which H_2O_2 positively regulates maturation (Chaube et al., 2005; Tripathi et al., 2009).

GSH levels change throughout oogenesis: they increase during maturation, and further increase as meiosis progresses, reaching the highest levels in metaphase II (two fold above levels in prophase I arrest). After meiosis is complete, GSH levels decrease significantly, reaching the lowest levels in the two cell embryo (Luberda, 2005) (**Figure 3.B**). Such large changes in GSH levels tightly associated with meiotic resumption may signify an important role of the GSH system in this process. It is possible that high levels of H_2O_2 produced by the mitochondria prior to maturation helps maintain the prophase I arrest. A decrease in H_2O_2 levels due to an increase in GSH brings H_2O_2 concentrations into the range promoting maturation (**Figure 3.B**).

In addition to ROS, reactive nitrogen species (RNS) such as nitric oxide (NO) are also reduced by GSH. NO is an important regulator of both cAMP and cGMP levels in mammals, both of which are secondary messengers that are important for egg maturation. High levels of NO inhibit meiotic resumption, and if prolonged, trigger apoptosis of rat oocytes. Conversely, a reduction in NO promotes meiotic resumption of diplotene arrested rat oocytes (Sela-Abramovich et al., 2008; Tripathi et al., 2009) **(Figure 3.B)**. Also, in *C. elegans*, the Major

Sperm Proteins (MSPs) that induce oocyte maturation and meiotic resumption activate RNS signaling pathways (Yang et al., 2010).

If the roles of ROS, RNS, and GSH in meiotic maturation and activation are conserved in Drosophila, the DHD thioredoxin, capable of glutathione reductase activity, could be involved in regulating GSH levels through the recycling of the reduced form of GSH (GSSG, after reaction with ROS or RNS) back into GSH.

Vitelline membrane crosslinking

The sV23 protein is a vitelline membrane (VM) protein that contains three canonical cysteine residues in a VM domain present in all vitelline membrane proteins (Wu et al., 2010). Disulphide bonds stabilize the interaction between vitelline membrane proteins, allowing for the hardening of the eggshell during activation so that the egg can withstand the mechanical pressure as it passes through the oviduct (Wu et al., 2010). Recently, it has been proposed that thioredoxins may be important for reducing VM cysteine residues and allowing for the crosslinking of the vitelline membrane that surrounds the oocyte, specifically within the sV23 protein (Wu et al., 2010). However, this would require DHD activity in follicle cells, the cells that are involved in the production of the vitelline membrane. Although in situ hybridization and protein-CFP experiments did not show evidence of *dhd* mRNA or protein expression in the follicle cells (Svensson et al., 2003), this does not conclusively rule out this hypothesis. The expression of *dhd* mRNA may have been too low to be detected through in situ hybridization, and the fact that the DHD-eCFP fusion protein was not functional means localization data from that experiment may not be reflective of endogenous DHD expression. Reassessing *dhd* expression using clonal analysis (discussed below) would be necessary to determine if DHD is active in follicle cells.

Future directions

Oxidation state specific imaging techniques (such as use of reducible fluorescent dyes) can provide insight into general redox changes during maturation of wild-type and *dhd* oocytes. If *dhd* oocytes show differences in redox state, redox specific mass spectrometry may be performed, which will allow the identification of proteins with different redox states by comparing the protein conjugates formed when reduced cysteines are modified in *dhd* and wild-type eggs. Monitoring GSH/GSSG levels throughout activation and maturation in wild-type and *dhd* oocytes can identify if there is a correlation between DHD activity and the ratio of alternate redox forms of GSH. This will evaluate if DHD is important for maintaining GSH pools in the developing oocyte. Also, performing a suppressor screen will further identify genetic interactions to help elucidate the pathways DHD is involved in during meiosis or oogenesis.

Further study of the role of DHD on vitelline membrane crosslinking will require clonal analysis in which only follicle cells express the *dhd* mutation. Characterizing these eggs to see if the *dhd* phenotype is observed would show whether DHD function is required in the follicle cells. Assaying for turgidity and dye permeability of these clonal *dhd* mutant eggs will allow the study of the putative role of DHD in VM crosslinking. The crosslinking of the sV23 protein in *dhd* eggs can also be assayed biochemically by measuring sV23 network formation. This can be done using His-tagged sV23 and Ni-affinity chromatography to isolate sV23 proteins in eggshell extracts, and comparing the ratios of high and low molecular weight sV23-his by immunobloting with anti-His antibody (method described in Wu et al. 2010). By comparing blots of *dhd* and wild-type eggs, the contribution of DHD activity to cysteine-bond dependent VM crosslinking will be identified.

II. Calcium regulation of egg activation in Drosophila

A conserved role for calcium in egg activation

The role of calcium in activation of oocytes has been well documented in vertebrates (including mammals) (Jones, 2005). As mentioned in the introduction, fertilization by sperm in mammals induces a wave of Ca^{2+} in the oocyte cytoplasm, activating CaMKII, which likely leads to degradation of the Emi2 inhibitor of the APC/C. The APC/C targets Cyclin B and Securin for destruction through ubiquitination, allowing for completion of meiosis II (Tatone et al., 2002; Jones, 2005; Madgwick et al., 2006; Backs et al., 2010; Li et al., 2011). The role of calcium in Drosophila meiosis and activation is now a topic of intense study, and it is beginning to emerge as one of the key regulators in egg activation. Pathways involving Calcineurin, Calmodulin and the APC/C in Drosophila may be involved in pathways similar to the Ca^{2+}-dependent signaling pathways in vertebrates.

sra, a regulator of Calcineurin, is required for Drosophila egg activation

Calcineurin (CN) consists of two subunits; the catalytic CnA subunit that is a kinase and binds Calmodulin (CaM) and Ca^{2+}, and the regulatory CnB subunit that binds Ca^{2+} (Klee et al., 1979; Rusnak and Mertz, 2000). Takeo et al. have shown that eggs without an active CnB subunit (loss-of-function mutation in the *CanB2* gene) do not complete meiosis and instead arrest in anaphase I. The *sarah (sra)* gene in Drosophila was identified through a screen for female-sterile mutants. Sra is a member of the class of proteins called Regulators of Calcineurin (RCANs), also referred to as Modulatory Calcineurin-Interacting Proteins (MCIPs), and it regulates CN activity by binding to CnA (Horner et al., 2006; Takeo et al., 2006, 2010). *sra* null eggs do not complete meiosis, mostly arresting at anaphase I (98%), the same phenotype as *canB2* eggs (Takeo et al., 2006). Since vitelline membrane crosslinking still occurs in *sra* eggs,

some aspects of activation are independent of this protein, but Sra function in the oocyte seems to be important for other characteristics events of activation, including Bicoid (Bcd) translation and decreasing Cyclin B levels (Horner et al., 2006; Takeo et al., 2006, 2010, 2012).

A model for the regulation of Calcineurin in Drosophila activation

Sra plays an endogenous role as a regulator of Calcineurin activity (Takeo et al., 2010, 2010). The current model proposed by Takeo et al. (2012) (see **Figure 4**) suggests that both CaM and Sra are associated with CnA in the oocyte prior to activation. Through phosphorylation by the MAPK pathway during oocyte development, Sra is phosphorylated at Ser219. This phosphorylation primes Sra for phosphorylation at a second site, Ser215. At activation, Ser215 is phosphorylated, and this phosphorylation is dependent on GSK-3β activity. There is, however, currently no evidence showing GSK-3β activity increases at activation. Ser215 phosphorylation is necessary to release the metaphase I arrest, possibly by changing the conformation of CnA. In addition to Sra, Ca^{2+} binding is hypothesized to be necessary for full CN activation. CaM would be activated by an increase in Ca^{2+} upon activation, and Ca^{2+} directly interacts with CnB, contributing to CN activation (Horner et al., 2006; Takeo et al., 2006, 2010, 2012).

Mechanical processes of activation involve Ca^{2+}

Activation of Drosophila eggs involves mechanical forces applied as they pass through the oviduct through hydrostatic and osmotic pressure. When stage 14 Drosophila oocytes are placed in hypotonic buffer, this causes swelling and activation, demonstrating osmotic pressure may be one factor that induces activation (Mahowald et al., 1983; Page and Orr-Weaver, 1997). Such osmotic pressure may cause activation through a mechanically-gated (MG) ion channels. Inhibition of MG channels with gadolinium inhibited activation *in vitro*, suggesting osmotic pressure triggers activation through a mechanically gated-response (Horner and Wolfner, 2008).

Hydrostatic pressure may also be important, as Horner and Wolfner demonstrated that pressure applied to the outside of the oocyte in a French press increases vitelline membrane hardening and protein translation (characteristic events of activation) (2008). Interestingly, external calcium is required for both hypo-osmotic and hydrostatic aspects of activation *in vitro*. It was proposed in this paper that these mechanical processes allow Ca^{2+} to enter the egg through MG ion channels, allowing Ca^{2+} to act as a second messenger within the cell to initiate activation signaling cascades. Furthermore, Takeo et al. (2012) hypothesized that these mechanical signals may be the upstream activators of GSK-3β, which in turn activates the Sra/Calcineurin complex. These potential roles for Ca^{2+} in Drosophila egg activation highlight a conserved role for this second messenger between species.

Future directions

Further identification and characterization of mechanically-gated and stretch-activated ion channels in the Drosophila oocyte, especially Ca^{2+} channels, will be helpful in determining whether the hypothesis of Horner and Wolfner (2008) is supported, and mechanical forces do allow for Ca^{2+} influx into the egg at activation. Identification of new Ca^{2+} channels in the oocyte may be accomplished using sequence analysis due to highly conserved domains in these proteins, followed by expression studies. Particular ion channel inhibitors, mutations in these channels, inhibitors of store-operated calcium (SOC) entry (release of Ca^{2+} from intracellular stores, i.e. the ER), and mutations in the genes involved in the proposed calcium-dependent activation pathway (*sra, Can* subunits, *CaM, Gsk-3β*, genes in the MAPK pathway) all will be helpful in assessing the role for calcium in activation *in vitro* and *in vivo*. Additional genetic interactions and biochemical techniques can be used to elucidate details of the signaling pathway and the unknown substrates of Calcineurin in activation. Real time intracellular Ca^{2+} monitoring (ratios

of free to bound calcium) in oocytes may be especially helpful to explore the role of this ion in oogenesis.

III. A large scale proteomic screen reveals candidates for oocyte maturation and egg activation

Rational and Approach

In our lab, a large-scale proteomic screen was performed to identify proteins that either increase or decrease during oocyte maturation and/or egg activation (Kronja and Orr-Weaver, unpublished). Since maturation occurs at stage 12, comparing the ratios of individual proteins between stage 10 and 14 egg chambers would identify proteins with significantly different expression levels pre- and post- maturation. The nurse cells and follicle cells surrounding the oocyte are present in stage 10, but during oocyte development, both the nurse cells and follicle cells undergo apoptosis and are no longer present in stage 14 (see **Figure 1A**). Therefore, proteins that decrease in the stage 10 versus 14 comparisons cannot be concluded to result from maturation, since these decreases could alternatively be attributed to the loss of the nurse and follicle cells.

Activation occurs when stage 14 oocytes pass through the oviduct and uterus and is independent of fertilization. Comparing stage 14 oocytes and laid, unfertilized eggs isolates proteins that increase or decrease during egg activation. Since unfertilized eggs arrest after the completion of meiosis but do not undergo the mitotic divisions of embryogenesis, proteins involved in controlling embryonic divisions would not be a part of this analysis.

In vitro dimethyl peptide labeling was performed on lysates from stage 10 egg chambers and stage 14 oocytes, as well as unfertilized laid eggs, to label proteins from samples with either regular hydrogen or deuterium (heavy hydrogen). These labeled extracts were subjected to LC/MS (liquid chromatography followed by mass spectrometry) to compare relative protein levels between two samples at a time.

The proteins identified as upregulated between stages 10 and 14 may be involved in either the process of maturation itself (promoting maturation), or alternatively, involved in activation, and upregulated prior to the beginning of activation as the egg prepares for activation. It is difficult to tease out whether the identified candidate genes upregulated between stages 10 and 14 are involved in maturation or activation, so all candidates are presented together.

Select groups of candidate proteins:

i) Calcium binding and transport: Scp1, CBP, CG10641, and Stim

As described above, the proposed role of Ca^{2+} appears to be in egg activation rather than in maturation in Drosophila. Sarcoplasmic Calcium-Binding Protein 1 (Scp1), Sarcoplasimc Calcium-Binding Protein (CBP), and CG101641 are all calcium binding proteins, with *CBP* expressed at the highest levels in the female ovary, *Scp1* expressed throughout both male and female adults, and *CG101641* expressed throughout various stages of development and organs in the adult (Cox, 1990; Kelly et al., 1997; Graveley et al., 2010). All three of these proteins are present at significantly higher levels in stage 14 oocytes compared with stage 10 oocytes (Kronja and Orr-Weaver, unpublished). These results are consistent with a role for Ca^{2+} in activation, if increases in these calcium-binding proteins are necessary prior to activation in order to prepare the oocyte for activation.

In addition, the Stromal Interaction Molecule protein (Stim), a component of the endoplasmic reticulum calcium transport system, is expressed at a significantly higher level in unfertilized oocytes compared with stage 14 oocytes, indicating a rise in protein levels during activation. The endoplasmic reticulum acts as a store for intracellular calcium. Ca^{2+} ions within the ER are released through store-operated calcium (SOC) entry, in which an external signal induces an initial rise in intracellular Ca^{2+}, which acts as a second messenger by activating calcium-release

activated calcium channels in the ER, further increasing intracellular Ca^{2+} levels and amplifying the signal. Stim acts as a calcium sensor that moves from the ER membrane to the plasma membrane after intracellular Ca^{2+} stores are exhausted (Williams et al., 2001; Roos et al., 2005; Zhang et al., 2005; Penna et al., 2008). If SOC entry emerges as a mechanism that controls Ca^{2+} levels at egg activation, Stim may be an important regulator.

ii) Steroid metabolism: Woc and CG7840

The precise external signals that initiate maturation in Drosophila are unknown, however, it has been proposed that hormone signaling involving prostaglandins or ecdysone may act in a role analogous to LH in mammals, triggering maturation and the progression of meiosis beyond the prophase I arrest point (Von Stetina and Orr-Weaver, 2011). The steroid hormone ecdysone induces maturation in *Locusta migratoriai* (locust) and *Dirofilaria immitis* (nematode) oocytes. In *L. migratoriai*, ecdysone levels increase when meiosis resumes both at maturation and activation. Furthermore, when locusts are fed diets designed to reduce ecdysone biosynthesis, eggs do not undergo maturation (Lanot et al., 1987, 1988). Incubation of immature oocytes with exogenous ecdysone initiates maturation in a dose-dependent manner in both *L. migratoriai* and *D. immitis*, strongly suggesting ecdysone is an upstream initiator of maturation in some insects and nematodes (Lanot et al., 1987; Barker et al., 1991).

Without Children (Woc), a protein that is involved in the ecdysone biosynthesis process (Wismar et al., 2000), was upregulated during activation, supporting a putative role of ecdysone in Drosophila meiotic resumption. Interestingly, the first mutated allele of *woc* that was identified caused female and male sterility (woc^{stl}), but the allele was not further characterized (Wismar et al., 2000). Since its initial characterization in the ecdysone synthesis pathway, evidence has shown *woc* is a transcription factor and also plays a role in telomere capping (Raffa

et al., 2005; Font-Burgada et al., 2008; Abel et al., 2009). Also, the uncharacterized gene *CG7840* encodes a 3-oxo-5-alpha-steroid 4-dehydrogenase (also referred to as 5-α reductase). This enzyme is involved in the biosynthetic pathway of testosterone in mammals, converting testosterone to 5-α-dihydrotestosterone (DHT) (Celotti et al., 1992; Penning, 2010). Its upregulation during maturation may signify a role of this gene in the modification of ecdysone or other steroids that play a role in signaling meiotic resumption in Drosophila.

iii) Ubiquitin pathway: Ubc-E2H, CG9636, CG6966, CG2924, CG500

A large number of proteins whose levels increase significantly during maturation (between stages 10 and 14) or activation (between stages 14 and the unfertilized egg) are involved in the ubiquitination pathway (Ubc-E2H, CG9636, CG6966, CG2924, and CG500). Ubiquitination is already known to be important in Drosophila activation; an ovary-specific meiotic APC/C regulator, Cortex (CORT), is crucial for some aspects of activation and the completion of meiosis (Lieberfarb et al., 1996; Page and Orr-Weaver, 1996; Pesin and Orr-Weaver, 2007). These newly identified genes may play a role in promoting the degradation of proteins maintaining either the prophase I or metaphase I arrest, allowing meiosis to resume at both maturation and activation points.

iv) Stress response genes: TotC and oxidative stress responders

Turandot proteins are regulators of the in the humoral stress response in Drosophila (Ekengren and Hultmark, 2001; Ekengren et al., 2001). One member of the Turandot protein class, TotC, is upregulated in egg activation. Unlike heatshock proteins, which respond to limited types of cellular stress, the Turandot proteins, including TotC, may be induced by a wide variety of stresses, including dehydration, and changes in osmotic pressure and mechanical pressure (Ekengren and Hultmark, 2001). Activation of Drosophila oocytes involves osmotic and

hydrostatic mechanisms through hydration and passage through the oviduct and uterus, respectively (Page and Orr-Weaver, 1997; Horner and Wolfner, 2008). It is possible that the pressure exerted on oocytes during activation induces *TotC* expression, which may lead to downstream signaling pathways.

Also, many oxidative stress response proteins are upregulated during maturation and activation. These include Alph (Alphabet), Whd/CPT1 (Withered), CG6084, which increase during maturation, and Heat Shock Protein 26 (Hsp26), which increases during activation. The possible role of DHD in oocyte maturation and activation was explored previously. If DHD and ROS are indeed involved in meiotic control, it is possible that other redox genes, including those involved in the oxidative stress response, are important during this stage.

v) Gap junctions: Zpg/Inx4

As mentioned previously, gap junctions play important roles in orchestrating communication between the oocyte and surrounding cells in a variety or organisms (including vertebrates and *C. elegans* (Von Stetina and Orr-Weaver, 2011). Von Stetina and Orr-Weaver hypothesized that Innexins, proteins that are a part of gap junctions in Drosophila, may play a role in oocyte-follicle cell communication during meiosis (2011). Evidence from this proteomic screen lends support to this hypothesis. Protein levels of Innexin-4, also known as Zero Population Growth (Inx4/Zpg) decrease during activation. Unlike other members of the Innexin family in Drosophila, Inx4 is found only in the membranes of nurse cells and the oocyte (both cells of germ-cell origin), not in the somatic follicle cells (Stebbings et al., 2002). Through immunohistochemistry, Inx4 has been shown to interact with another member of the Innexin protein family, Inx2, which is found in the membranes of the follicle cells, as well as the nurse cells and the oocyte (Bohrmann and Zimmermann, 2008). This Inx4/Inx2 interaction may be part

of a gap junction between the oocyte and follicle cells, providing a channel of communication between these cells. Inhibition of Inx2 affects oocyte growth and development, while mutations in *inx4* cause defects in germ cell survival and differentiation, but the roles of Inx2 or Inx4 in meiosis has not been studied (Tazuke et al., 2002; Gilboa et al., 2003; Bohrmann and Zimmermann, 2008). The change in Inx4 levels during activation may indicate a function of gap junctions and intercellular communication in meiotic regulation. For example, if gap junctions allow for the transfer of an inhibitory molecule between the follicle cells and oocyte, the decrease in Inx4 upon activation may decrease this signaling and allow for meiotic resumption.

Future directions

Since this was a large-scale study, further verification is required to ensure that the levels of the identified candidate proteins do indeed change during maturation or activation. If antibodies are currently available against candidate proteins, this verification is possible through western blot. Many Drosophila stocks are available with mutations within these identified genes, or with constructs inducing germline specific RNAi (Bloomington Drosophila Stock Center), to permit knockdown of gene products in the germline. These mutant stocks will be assessed for sterility and failure to progress properly through meiosis, which may be visualized by staining the DNA of unfertilized eggs with DAPI or propidium iodide and examining these eggs for evidence of meiotic completion. If mutations in these genes do indeed cause meiotic arrest due to inability to initiate or complete maturation and activation, further characterization of these phenotypes (biochemical/cytological) and genetic and protein interactions will help identify the role they play in controlling meiosis.

Conclusions

Despite the intense study of Drosophila meiosis to date, many aspects of meiotic control have yet to be clarified. New molecular, genetic and biochemical techniques are making in depth study of the regulation of activation and maturation possible. Elucidating the roles for *dhd*, Ca^{2+}, ubiquitination, steroid synthesis and signaling, gap junctions, and stress response genes may provide us with a deeper understanding of the genes, processes, and signaling pathways that regulate oocyte arrest and release throughout the stages of meiosis. Known similarities between mammalian/vertebrate meiosis and Drosophila meiosis demonstrate the conserved nature of meiotic regulation and chromosome separation between species. As more is known about meiotic regulation in Drosophila, we see that many of the new pathways identified as important in this organism also play a role in other organisms. Since Drosophila is ideal for performing basic research on oogenesis, due to ease of genetic manipulation, maintenance, mating control, oocyte production volume, etc., studying conserved processes in this organism can broaden knowledge of meiotic regulation in general, and ultimately enhance our understanding of factors controlling human fertility, reproduction, and development.

Figures

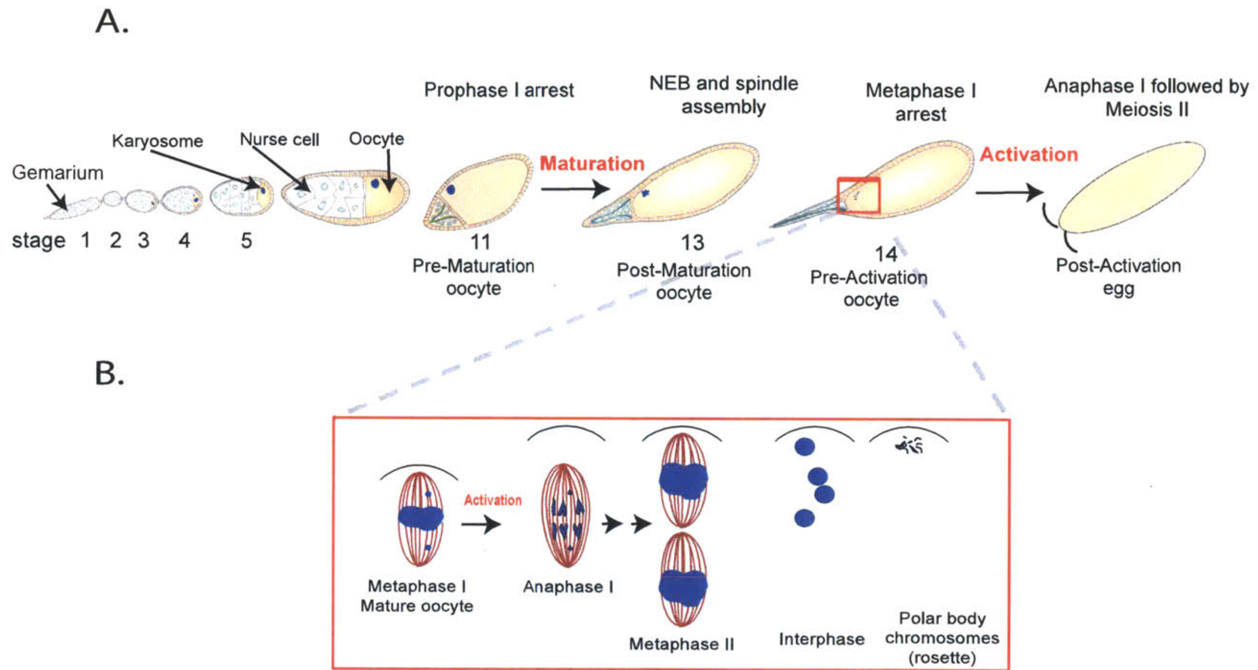


Figure 1. Meiosis in Drosophila. (A) Meiosis begins in the germarium, and arrests in prophase I with the chromosomes condensed into the karyosome. The nurse cells become polypoloid and increase in size, producing the mRNA that will be necessary to control the first mitotic divisions in the zygote. Maturation releases the prophase I arrest, and meiosis continues through metaphase I, at which point it arrests for a second time. Passage of the egg through the uterus causes activation, which induces complete meiosis independent of fertilization. Adapted from Xiang et al. (2007) by Iva Kronja **(B)** Movement of the chromosomes from the metaphase I arrest through the completion of meiosis. After meiosis is complete, the polar body chromosomes (the chromosomes from the unused meiotic products) form the characteristic 'rosette' pattern. Figure by Iva Kronja.

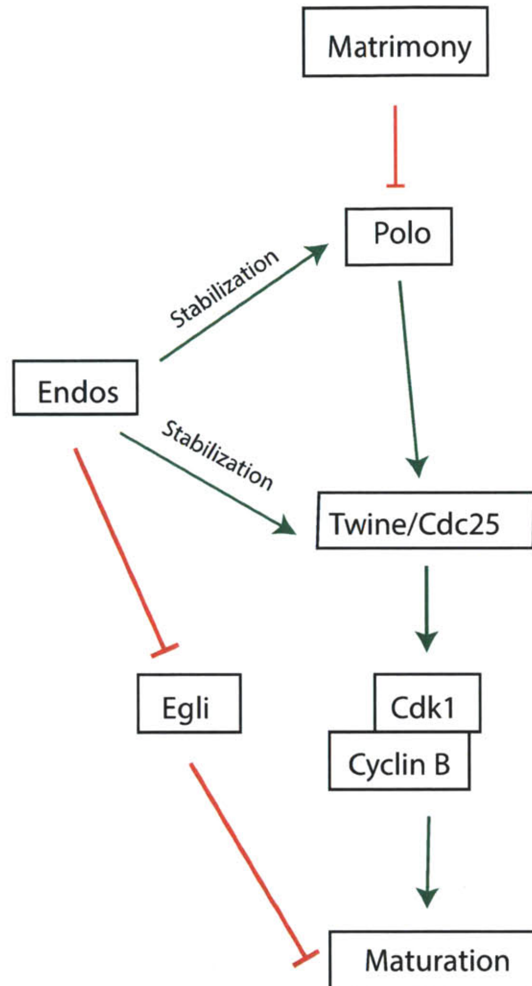
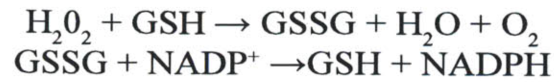


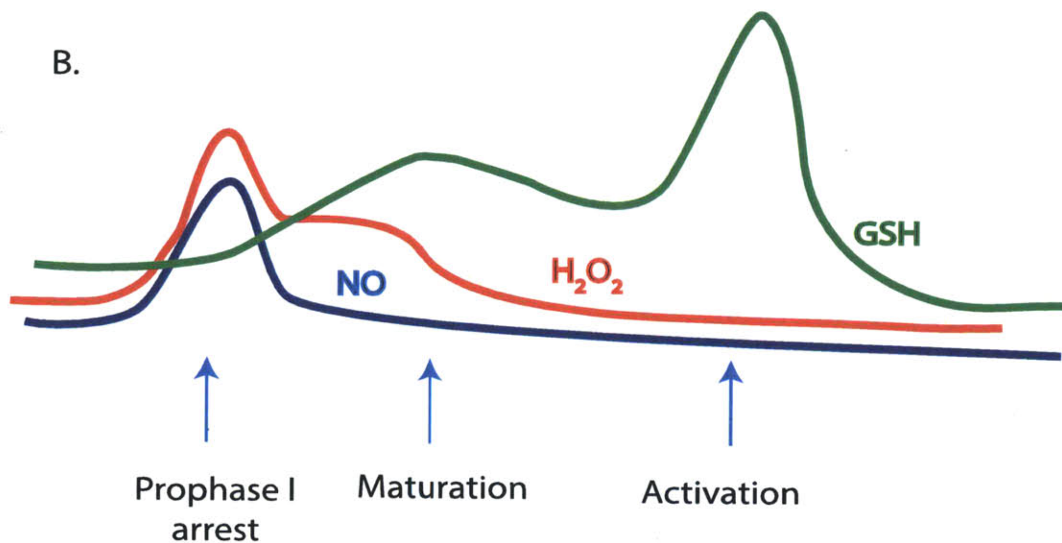
Figure 2. Control of Maturation in Drosophila. Matrimony binds to and inhibits Polo. When levels of Polo rise, excess active Polo can activate Twine/Cdc25, which in turn activates CyclinB through phosphorylation of Cdk1. This increase in Cyclin B allowing for maturation. Endos stabilizes Polo and Twine, and inhibits Egli, overall promoting maturation. Adapted from Von Stetina and Orr-Weaver (2011).

A.



$$H_2O_2 + GSH \rightarrow GSSG + H_2O + O_2$$
$$GSSG + NADP^+ \rightarrow GSH + NADPH$$

B.



	Conc.	Effect	Reference(s)
H_2O_2	High	Inhibits maturation: germinal vesicle breakdown and extrusion of first polar body	Chaube et al., 2005, 2008, 2009; Tripathi et al., 2009
	Low	Promotes maturation	Chaube et al. 2005, Tripathi et al., 2009
NO	High	Inhibits maturation	Sela-Abramovich et al., 2008; Tripathi et al., 2009

Figure 3. A model for RNS, ROS and GSH regulation in maturation and activation. (A) The redox pathway of GSH. **(B)** Levels of NO, H_2O_2, and GSH in meiosis. H_2O_2 shows a concentration dependent effect on maturation; high concentrations inhibit maturation while low concentrations promote maturation, while NO has only been shown to inhibit maturation. GSH levels rise throughout meiosis, reaching their highest levels at metaphase II, then decrease substantially in the embryo. The increase in GSH during maturation may decrease H_2O_2 and NO levels to concentrations condusive to maturation.

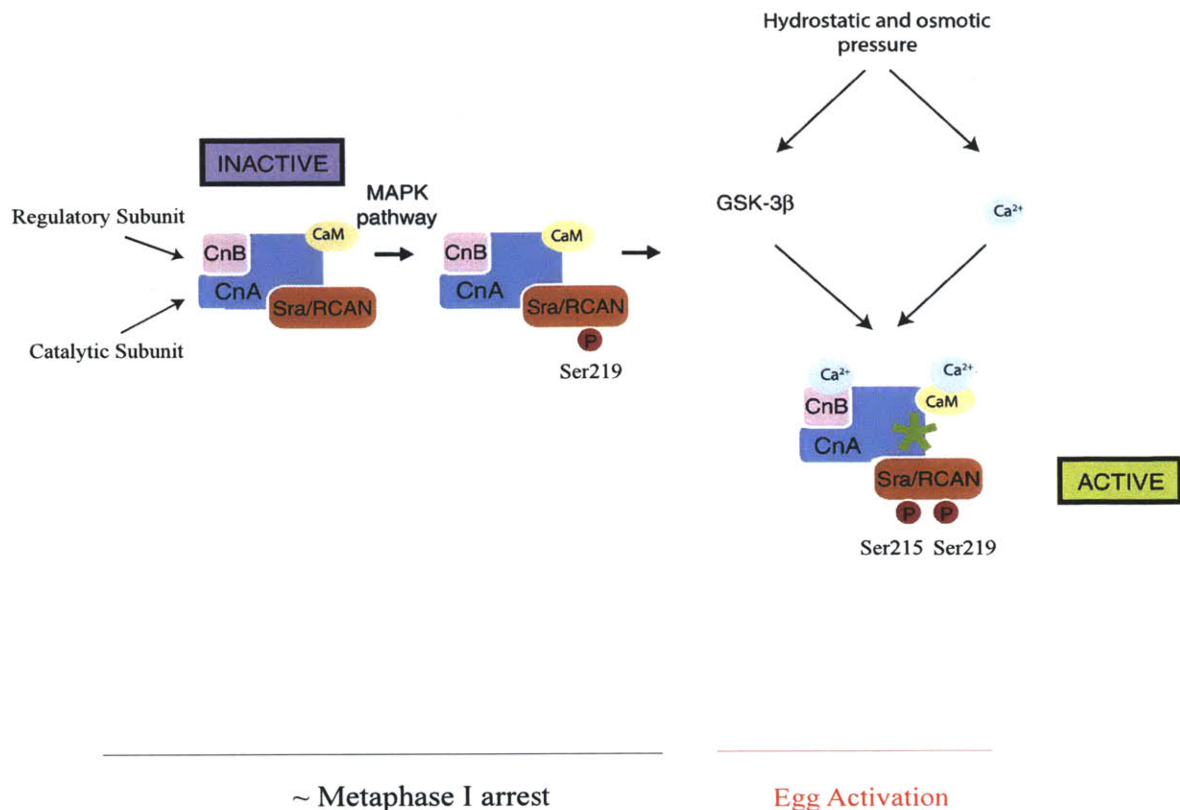


Figure 4. A model for the regulation of calcineurin by Sra, Ca^{2+}, and CaM. Both Sra and CaM are bound to the CnA subunit of Calcineurin prior to egg activation. Phosphorylation of Sra by the MAPK pathway at Ser219 primes Sra for further activating phosphorylation. GSK-3B becomes activated at egg activation, possibly by pressure exterted as the egg passes through the uterus, and phosphorylates Sra at Ser215, activating Sra. Ca^{2+} also enters the cell through stretch-activated and mechanically-gated Ca^{2+} channels, which open due to the hydrostatic and osmotic pressure applied to the egg during this time. Ca^{2+} binds to both the CnB regulatory subunit and CaM, which is bound to CnA. These changes result in a conformational change of CnA, activiating its kinase activity and allowing for the completion of meiosis through downstream signalling. Modified from Takeo et al., 2010, model proposed by Takeo et al., 2010, 2012.

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