PHOSPHORYLATION CHANGES DURING EGG ACTIVATION AND ON REGULATORS OF THE FIRST EMBRYONIC CELL CYCLE IN DROSOPHILA

A Dissertation

Presented to the Faculty of the Graduate School

of Cornell University

In Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

by

Katharine Lillington Sackton

January 2009

PHOSPHORYLATION CHANGES DURING EGG ACTIVATION AND ON REGULATORS OF THE FIRST EMBRYONIC CELL CYCLE IN DROSOPHILA

Katharine Lillington Sackton, Ph. D.

Cornell University 2009

At the transition from oocyte to embryo, a quiescent mature oocyte is transformed in a short span of time to a rapidly dividing embryo. In the first two hours of *Drosophila melanogaster* embryogenesis there is very little transcription of the zygotic genome, so regulation of translation of maternally-loaded mRNAs and post-translational modifications are the primary mechanisms of controlling the events of the oocyte-to-embryo transition. Changes in protein phosphorylation occur during egg activation, and I have investigated several aspects of these phosphorylation changes by testing the activity/or and roles of MAP kinases, and PP1 and MKP3 phosphatases during this developmental transition. I also examined the relationship between these kinases/phosphatases, four genes known to be necessary for egg activation (*sarah, cortex, prage*, and *wispy*), and two proteins (YA and GNU) that are dephosphorylated upon egg activation and act to initiate or modulate embryonic mitosis. I further defined the cell-cycle arrest point of one of these latter phosphoproteins, YA.

Changes in phosphorylation state are likely to be important for regulating multiple aspects of the oocyte to embryo transition. My studies have shown that MAP kinases are dephosphorylated upon egg activation. In addition, the egg activation genes *cort*, *sra,* and *prg* are upstream of YA dephosphorylation upon egg activation, and *cort* and *sra* are also upstream of GNU dephosphorylation. I identified the phosphorylation sites on YA from oocytes and embryos by mass spectrometry. During oocyte maturation, *dmos*, *Ya* and *gnu* mRNA polyadenylation and protein levels are

regulated by *wispy*, which also regulates ERK and JNK phosphorylation in oocytes, probably through *dmos*. Testing the roles of phosphatases, I have shown that PP1s may have a role in egg activation, but MKP3 is primarily needed after egg activation, prior to the first mitosis. I also clarified the time of the cell cycle when YA is necessary; YA acts after egg activation to regulate the entry into mitosis after the first S phase.

BIOGRAPHICAL SKETCH

Kate grew up in Shrewsbury, Massachusetts, with her parents and younger brother, Nancy, Matthew, and Andrew Beach. She attended Shrewsbury public schools, was a voracious reader, and excelled academically. She attended Brown University and during her undergraduate years juggled classwork, her work-study job in the main dining hall, her senior research project in virology in the lab of Walter Atwood, and membership in the band, an acapella group, and the Alpha Delta Phi Society. Kate graduated in 2000, but stayed in Providence as an Americorps*VISTA, working at the Mount Hope Learning Center to renovate an old house into a public space for after school programs in literacy, art, and technology. She then spent a year as a technician at Harvard Medical School in the lab of Randy King, studying a small molecule inhibitor of exit from mitosis (the metaphase to anaphase transition). In August 2002, Kate moved to Ithaca with Tim, in the third year of their relationship. Kate continued to study the cell cycle in the Wolfner lab, focusing on the YA protein that acts at the transition from completion of meiosis to the first mitosis. During the same period of time as Kate's graduate studies, Tim did his PhD in the field of Ecology and Evolutionary Biology studying the evolution of Drosophila immunity in the lab of Andy Clark. In July 2004 Kate and Tim got married in their home state of Massachusetts. After Cornell, Kate and Tim will do postdocs in Boston, and enjoy being near family.

ACKNOWLEDGMENTS

My advisor Mariana Wolfner has taught me about research, collaboration, mentoring and teaching, and provided a wonderful example of a true participant in multiple scientific communities. The members of the Wolfner Lab have been a great scientific and social support, especially my baymate Norene Buehner and the YActivation group members Vanessa Horner, Jun Cui, and Amber Krauchunas. I thank my committee members Mark Roberson and Lee Kraus for their helpful comments and suggestions of reagents and experiments over the years.

I would also like to thank Cornell's support facility staff. Carol Bayles in the Microscopy and Imaging Facility (MIF) provided helpful training and troubleshooting advice on the confocal microscope. Sheng Zhang and Sabine Baumgart did mass spectrometry experiments and taught me to analyze the results. I am grateful to all the faculty for whom I was a teaching assistant: Volker Vogt, Jim Blankenship, and Susan Ealy, each a unique example of teaching, grading, and interactions with students.

I am grateful to my family for their support. Specifically, my husband Tim has made these years in Ithaca wonderful and memorable, and helped me scientifically as well as in other aspects of life. My parents, my extended family, and Tim's family have contributed to my well-being as long as I've known them, including these long-distance years in Ithaca.

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CHAPTER ONE

INTRODUCTION

Embryo development cannot occur without the prior events of egg activation, which transition a quiescent mature oocyte into a rapidly-developing embryo. In *Drosophila melanogaster* oogenesis many proteins and mRNAs are maternally provided to the egg in quantities that will supply the first fourteen cell cycles (about the first two hours of embryogenesis). There is very little transcription of the zygotic genome during this time in Drosophila, so regulation of translation and post-translational modifications on pre-existing maternally loaded molecules are the primary mechanisms of controlling the events of the oocyte-to-embryo transition. Protein phosphorylation can, in principle, occur at a suitably rapid rate to regulate at least some of the many molecular and physiological changes that constitute egg activation.

1.1 Oogenesis, oocyte maturation, and egg activation

In the Drosophila ovary, oocytes are continually produced from the stem cells at the tip of the ovaries (reviewed in Spradling 1993). The stem cells are located at the tip of each ovariole, a region called the germarium. The many ovarioles comprise "assembly lines" of oogenesis. Sixteen-cell cysts are produced in the germarium. One cell in the cyst will become the oocyte. The other fifteen cells will be nurse cells, which provide RNAs and proteins to the oocyte. The development from the sixteen cell cyst to a mature oocyte has been classified into fourteen stages, stage one being the exit from the germarium. At stage eight the yolk proteins begin to be taken into the oocyte, and the oocyte grows rapidly. By stage ten the nurse cells begin to break down

and “dump” their contents into the oocyte. In stages nine to eleven synthesis of the oocyte vitelline membrane begins, and a chorion is laid down around it in stages ten to fourteen. Both components of the eggshell are secreted by surrounding follicle cells.

The oocyte nucleus is arrested in prophase I of meiosis from stages 1-12, then begins metaphase I in stage 13 and arrests in metaphase of meiosis I in the mature (stage 14) oocyte. Stages 1-7 take about two days, and stages 8-14 about one more day. The transition from prophase I to metaphase I from stage 13 to stage 14 is a characteristic of the process of oocyte maturation; another characteristic of this process is polyadenylation of certain mRNAs such as *cort* (Tadros et al. 2005; Pesin et al. 2007; Benoit et al. 2008). The overall pattern of cell cycle arrest in meiosis during oocyte development, then release of the mature oocyte from meiotic arrest by egg activation, is common to all metazoans. A more specific pattern of oocyte development during a prophase arrest, followed by resumption of meiosis during maturation and then arrest as a mature oocyte at a later cell cycle stage is typical of many animals including Drosophila, but the stage of mature oocyte meiotic arrest is species-specific.

My investigations of egg activation in Chapter 4 lead to observations regarding phosphoproteins and the MAPKs in oocyte maturation. The kinase Mos is necessary for establishing Xenopus's metaphase II meiotic arrest, functioning as an element of cytostatic factor (CSF) (Sagata et al. 1989), but in Drosophila *dmos* null mutants there is no effect on meiotic arrest and subsequent progression (Ivanovska et al. 2004). DMOS acts upstream of MEK and ERK in Drosophila oocytes, and its level decreases in embryos, as in Xenopus, but its downstream roles in oocyte maturation and or egg activation are different and/or redundant with another pathway. Another component of Xenopus CSF activity is inhibition of the APC by Emi2 (Tung et al. 2005), which is highly regulated by numerous pathways, including Mos-dependent p90rsk (Inoue et al.

2007; Nishiyama et al. 2007) and calcium-dependent pathways (Liu et al. 2005; Rauh et al. 2005; Nishiyama et al. 2007).

Egg activation is the series of events that occur when the mature oocyte transitions to an early embryo. The defining cell cycle transition of egg activation is the resumption of meiosis by the oocyte, but the process of egg activation includes additional events that occur at this time (reviewed in Horner et al. 2008b). The egg activation events described below as they occur in Drosophila are parallel to the egg activation events in other organisms, with some differences, such as the fact that egg coverings and their activation-induced changes vary between species. In Drosophila the egg activation events include, besides meiosis resumption, (a) physical changes such as hardening of the eggshell due to crosslinking of vitelline membrane proteins (Heifetz et al. 2001) and (b) (unrelated) cytoskeletal (microtubule) reorganization (Page et al. 1996; Theurkauf et al. 1992). In addition (c), translation of certain mRNAs (such as the patterning genes *toll, torso*, and *bicoid*) occurs, some of which is dependent on (d) mRNA polyadenylation, to produce proteins needed for early embryogenesis (Sallés et al. 1994). Other mRNAs (such as the cdc25 ortholog *string*) (Edgar et al. 1996; Tadros et al. 2005) are destabilized/degraded either to regulate their localization or to eliminate transcripts that are no longer needed. The *smaug* gene is regulated at the level of mRNA translation and the SMAUG protein is a regulator of degradation of mRNA upon egg activation, because SMAUG protein is translated upon activation, and it then acts in two different complexes to repress translation and to promote transcript deadenylation and destabilization (Semotok et al. 2005; Smibert et al. 1996; Tadros et al. 2007). Recent proteomic studies have described global changes in both protein levels and protein phosphorylation (V. Horner, A. Krauchunas, and M. Wolfner, unpublished) during Drosophila egg activation. These studies are identifying new genes that are translated or proteins that are dephosphorylated upon

egg activation in addition to those genes/proteins previously known to be so regulated in Drosophila.

The initial signal for egg activation in Drosophila appears to be an increase in intracellular calcium (Horner et al. 2008a, 2006, 2008b). Although calcium also triggers activation in other organisms (Ducibella et al. 2006), in vertebrates and marine invertebrates the cause of the increased calcium is the sperm. In contrast, in Drosophila and other insects, egg activation is independent of fertilization (Heifetz et al. 2001; King et al. 1970; von Borstel 1957; Went et al. 1974), whereas in other organisms it is fertilization that triggers the calcium signal. A calcium influx is thought to be triggered in Drosophila by mechanical stimulation and also possibly by a change in extracellular environment during ovulation (Horner et al. 2008a; Heifetz et al. 2001). Thus despite the differences in trigger, the calcium influx is the initial signal for events of egg activation both in Drosophila and other organisms. Drosophila genetics provide an excellent system to identify molecular components of the signaling pathways downstream of calcium, and to distinguish fertilization-independent aspects of egg activation from other developmental changes that occur due to fertilization.

Drosophila screens for female-sterile mutants have identified several genes whose mutants arrest during egg activation:

Sarah (*sra*) mutants arrest in anaphase of meiosis I, and also have defects in mRNA polyadenylation and translation of *bicoid* (Horner et al. 2006; Takeo et al. 2006). The *sarah* gene encodes the Drosophila calcipressin (also called RCAN1, MCIP1, *DSCR1*, or *Adapt78*), a regulator of calcineurin phosphatase. Calcineurin could regulate cell cycle progression upstream of the anaphase promoting complex/cyclosome (APC/C) and its regulator cdc20 through one or more possible targets. Calcineurin could act by regulating CaMKII, which activates the APC/C by

inhibiting the APC/C-inhibitor Emi2 (Liu et al. 2005), or calcineurin could regulate cdc20 directly (Horner et al. 2006; Mochida et al. 2007). Calcineurin also regulates sperm aster formation and thus pronuclear migration through as-yet-unknown molecular targets (Horner et al. 2006; Nishiyama et al. 2007). SRA is a phosphoprotein, but the regulation and developmental timing of its phosphorylation are not yet known (S. Takeo, personal communication).

Cortex (*cort*) mutants arrest in metaphase of meiosis II, as do mutants of *grauzone*, a transcription factor required for *cortex* transcription (Chen et al. 2000; Harms et al. 2000; Page et al. 1996). *Cortex* mutants also have defects in mRNA polyadenylation, translation and degradation (Lieberfarb et al. 1996; Tadros et al. 2003), and microtubule depolymerization (Page et al. 1996). Because *cortex* encodes a Drosophila cdc20, a regulator of the APC/C (Chu et al. 2001), it regulates levels of cyclin B in oocyte meiosis (Swan et al. 2007), which may be responsible for some of its cell cycle phenotype.

The exact cell cycle arrest point of *prage (prg)* mutants is not known, but *prage* mutants arrest during or around the time of meiosis, and they have defects in mRNA translation and degradation (Tadros et al. 2003). *Prage*'s molecular identity is also not known.

The *wispy* (*wisp*) gene encodes a poly(A) polymerase (PAP) of the GLD-2 family, and has meiotic defects and a final arrest right at the completion of meiosis (Brent et al. 2000; Cui et al. 2008; Benoit et al. 2008). GLD-2 family poly(A) polymerases are different from canonical PAPs because they lack an RNA-binding domain and thus are thought to interact with an RNA-binding protein in order to function (Wang et al. 2002). Because *wisp* is a poly(A) polymerase, *wisp* mutants have defects in mRNA polyadenylation and translation, and also have defects in mRNA destabilization (Tadros et al. 2003). Because *wispy* has significant roles during oocyte

maturation, such as polyadenylation of *Mos* mRNA (Cui et al. 2008), and also functions earlier in oogenesis (Benoit et al. 2008), it is not considered here to be solely an "egg activation gene" like *cortex*, *sarah*, and *prage*, but is addressed separately. The "egg activation genes" are part of the regulatory pathways that connect the initial calcium trigger of egg activation to the characteristic downstream events, but identifying the molecular interactions that make up these pathways is an area of current research that will lead to a more complete understanding of egg activation.

1.2 Fates of meiotic products after egg activation and fertilization

When Drosophila meiosis resumes the meiosis I spindle, which was parallel to the egg's long (anterior/posterior) axis, rotates to be perpendicular to that axis. Meiosis proceeds through anaphase I without further DNA replication, and then proceeds through meiosis II, forming a line of four female haploid meiotic products which remain in the egg (Endow et al. 1998). For a diagram illustrating the movements of meiotic products, see (Page et al. 1997). When sperm fertilizes the Drosophila egg the paternal chromatin is tightly condensed (sperm are specialized to be small and motile). Through the action of both paternal proteins and maternal gene products, the male pronucleus decondenses its chromatin and forms the male pronucleus; it is surrounded by a maternally provided nuclear envelope (Liu, Lin, et al. 1997). In wildtype embryos, the sperm pronucleus comes into close proximity to one female haploid meiotic product, usually the innermost, and that becomes the female pronucleus. The other three female meiotic products become the polar bodies, moving to the periphery of the egg and eventually combine and condense their chromatin into a "mitotic-like state" forming a characteristic rosette structure also called a polar body (Riparbelli et al. 2000).

Genetic screens have identified maternally provided (*maternal haploid, Hira/ssm,* and *chd1)* (Loppin et al. 2001; Loppin, Bonnefoy, et al. 2005; Konev et al.

2007) and paternally provided (*sneaky, misfire, pal* and *ms(3)K81)* (Wilson et al. 2006; Yasuda et al. 1995; Ohsako et al. 2003; Baker 1975) molecules that are needed for the conversion and function of the male nucleus. Their mutant phenotypes affect the paternal chromatin, paternal nuclear envelope, or paternal pronuclear migration. In eggs from these maternal effect mutants or eggs fertilized by these paternal effect mutants' sperm, the female pronucleus and other female meiotic products proceed as they normally would, initiating the meiotic cell cycle and then procedeing through the normal thirteen maternally-driven mitotic cycles-- but with haploid nuclei (haploidy in *D. melanogaster* is eventually lethal).

The first mitotic division in Drosophila is a gonomeric division (Kawamura 2001). The male and female pronuclei become apposed, and their nuclear envelopes partially disassemble to form “spindle envelopes” which are open at the poles. They replicate their DNA and their chromosomes condense coordinately into two separate mitotic plates. The spindle envelope allows independent parallel spindles to nucleate from the maternal and paternal chromosome groups. There is no mixing of the parental genomes until the synchronous telophase results in the formation of two zygotic nuclei. In Drosophila the first fourteen mitotic divisions take place rapidly, with no (or minimal) gap phases, and no cytokinesis. The nuclei in an embryo cycle in approximate synchrony between S phase and M phase in a common cytoplasm, called a syncytium (Foe 1993). At the end of the fourteenth cycle, cell membranes grow down between the nuclei. This cellularization process occurs about two hours after fertilization.

1.3 Phospho-regulation, kinases and phosphatases

Two Drosophila proteins are known to be dephosphorylated upon egg activation. Both YA (described in section 1.4 below) and GNU (giant nuclei) have roles in regulating early embryonic mitosis (Freeman et al. 1987; Lin et al. 1991).

GNU is a component of the Pan Gu (PNG) kinase complex (Lee et al. 2003). Pan Gu kinase activity requires its association with the plutonium (PLU) protein, an association mediated by GNU. Null mutation of any of the three maternal-effect genes in the complex results in the same phenotype, an uncoupling of the syncytial S and M phase cycles resulting in repeated rounds of DNA replication (Freeman et al. 1987; Shamanski et al. 1991). Therefore giant nuclei are formed in activated, unfertilized eggs and in embryos laid by these mutant females. Function of PanGu kinase genes must be needed early after egg activation because no giant nuclei form in *gnu*$^{-}$, *plu*$^{-}$, or *png*$^{-}$ mature oocytes (or earlier). PanGu is a Ser/Thr kinase that phosphorylates GNU, PLU and a novel cell-cycle related protein, Mat89Bb, in vitro (Fenger et al. 2000; Lee et al. 2005). Cyclin B is regulated downstream of the PNG kinase complex, based on decreased cyclin B levels in all three mutants, and genetic interactions, but cyclin B is not a substrate of PNG kinase in vitro (Lee et al. 2001, 2003).

GNU is phosphorylated in oocytes, and dephosphorylated in activated, unfertilized eggs and in embryos (Renault et al. 2003). GNU has seven predicted MAPK target sites. S/T to A mutations in three of these sites together are sufficient to increase the electrophoretic mobility of oocyte GNU so that it is more similar to embryo GNU (Zhang et al. 2004). Thus GNU is likely a MAPK substrate in oocytes. Dephosphorylation of GNU also occurs in oocyte extracts in the absence of phosphatase inhibitors. Comparison of the effectiveness of various phosphatase inhibitors in inhibiting GNU dephosphorylation in vitro shows that GNU is dephosphorylated by PP1 phosphatases more than by PP2A phosphatases. The slow-mobility (phosphorylated) form of GNU is still present in *png* mutant ovaries, suggesting that PNG is not the primary kinase that phosphorylates GNU in oocytes.

Kinases: The mitogen-activated protein kinases (MAPKs) play essential roles

during oocyte maturation and egg activation and are also active in somatic cell cycle regulation in many animals. In clams, starfish, ascidians, mice, and frogs, the species-specific timing of MAPK activity during oocyte maturation and egg activation, as assayed biochemically by the ability of extracts to phosphorylate the common MAPK substrate MBP, correlates with the different meiotic arrest points of these various organisms (Whitaker 1996). Furthermore, MAPKs have been shown to regulate the meiotic cell cycle in marine invertebrates and vertebrates downstream of Mos and through Emi2 (Nishiyama et al. 2007). It was not previously known whether changes in MAPK activity accompany egg activation in insects. I have shown that levels of phospho- (active) MAPKs decrease upon egg activation in Drosophila (Chapter 2, Sackton et al. 2007).

MAPKs are said to be part of phosphorylation cascades because at least two kinases transduce an initial signal by sequential phosphorylation upstream of the MAPKs, which then phosphorylate their targets (Roux et al. 2004). Diagrammatically this can be summarized as:

SIGNAL--> MAPKKK--> MAPKK--> MAPK--> SUBSTRATE.

In general, there are three main classes of MAPK: ERK, JNK, and p38. The Drosophila genome contains only five MAPK genes (Morrison et al. 2000), *rolled* (*rl*, ERK), *basket* (*bsk*, JNK), *p38b* and *Mpk2* (both p38 MAPKs), and *CG2309* [ERK5/BMK1, a less-studied class (Chang et al. 2001)]. Of the two p38 kinases, is more highly expressed in oocytes than based on semi-quantitative PCR (C. Sartain, unpublished data). There is no expression data regarding the presence/absence of *CG2309* in oocytes or early embryos. The MAPKs' activities are regulated by MAPK kinases (MAPKKs). In Drosophila the ERK kinase (MEK) gene is *downstream of raf 1* (*dsor1),* the p38 kinase gene is *licorne* (*lic,* MKK3), and the JNK kinase gene is *hemipterous* (*hep,* MKK7). Dual phosphorylation of MAPKs on a conserved Thr-X-

Tyr motif in the activation loop by these upstream kinases activates their kinase function. A constitutively active lesion of *rl*, the Sevenmaker allele (rl^{Sem}) (Brunner et al. 1994), has been proposed to block dephosphorylation of the activation loop, therefore maintaining the active, phosphorylated state of *rl* (ERK) (Bott et al. 1994; Chu et al. 1996). MAPKs are proline-directed serine/threonine kinases, and their consensus target phosphorylation site motif is Pro-X_{1-2}-Ser/Thr-Pro (Roux et al. 2004). The specificity of different classes of MAPKs comes in part from recognition of docking sites on substrates (Jacobs et al. 1999), but specificity is not always absolute, and MAPKs' activities may be redundant in some circumstances.

Other kinases known to be active in oocytes and embryos include polo, GSK3, and CAMKII. CaM-protein kinase II (CAMKII) is of interest because it is calcium-regulated, It has been shown to regulate cell-cycle resumption in Xenopus by inhibiting the APC/C-inhibitor Emi2, thus activating the APC/C which degrades cyclin B, promoting the metaphase to anaphase cell cycle transition and poly(A) polymerase activity (reviewed in Ducibella et al. 2008). Preliminary data in Drosophila suggest that the phospho- (active) form of CaMKII is present in oocytes and dephosphorylated upon egg activation (V. Horner, unpublished results). Polo kinase also regulates Emi2 in Xenopus (Liu et al. 2005). Bioinformatic analysis predicts that these two kinases phosphorylate sites in YA that I have shown by mass spectrometry to be phosphorylated in oocytes and embryos (Chapter 3). Drosophila *polo* mutant embryos arrest before the first mitosis with defects in meiosis, polar body arrest, sperm aster formation, and pronuclear migration (Riparbelli et al. 2000). These phenotypes are related to *polo*'s function in regulating microtubule organizing centers (MTOCs). Bioinformatic analysis also predicts that GSK3 phosphorylates an oocyte-specific site in YA detected by mass spectrometry. A Drosophila GSK3-beta kinase that functions in embryogenesis is shaggy (*sgg*), a component of the Wingless, Wnt

and Hedgehog signaling pathways (Jia et al. 2002).

Phosphatases: The activity of kinases is opposed by phosphatases. Phosphatases include Ser/Thr phosphatases such as PP1 and calcineurin, and Tyr or dual-specificity phosphatases such as MAP kinase phosphatases (MKPs) and twine and string (cdc25 orthologs). Of the many phosphatases in Drosophila, the above are candidates for functioning in egg activation based on previous studies described below. There are four genes for PP1 catalytic subunits in the Drosophila genome; they have redundant functions (Kirchner et al. 2007). Despite this redundancy, they are of interest to assay in egg activation because PP1 dephosphorylates GNU in oocyte extracts (Renault et al. 2003). The PP1 class of phosphatases can be inhibited in vivo by over-expression of the PPI negative regulators Nipp or Inhibitor 2 (I2) (Bennett et al. 2003). Calcineurin is a calcium-regulated phosphatase that is also regulated by *sarah,* and in Drosophila*, sarah* is required for egg activation, suggesting that calcineurin functions in egg activation. Calcineurin has been shown to be involved in egg activation in Xenopus (Nishiyama et al. 2007). Dual-specificity phosphatases (DSPs) are important to consider because they can dephosphorylate MAPKs, which are regulated during egg activation. One DSP in Drosophila is MKP3. Germline clones of the Drosophila MKP3 arrest as embryos with fewer than five nuclei (Kim et al. 2004 and Chapter 3). *Twine* is a female-sterile gene required for meiosis (White-Cooper et al. 1993). Twine is a cdc25 homolog, which helps activate cyclin-dependent kinase by removing an inhibitory phosphorylation. String is a cdc25 homolog that functions similarly in embryonic mitosis, and these two cdc25 phosphatases are somewhat redundant (Edgar et al. 1996). DSPs that already have important identified functions in oocytes and embryos (MKP3 and cdc25s) might also play a role in dephosphorylation upon egg activation of MAPKs (especially by MKP3) or other Tyr-phosphorylated proteins.

1.4 A protein downstream of egg activation: Young Arrest (YA)

The maternal effect sterile gene *fs(1)Ya* encodes a phosphoprotein necessary for the first mitosis (Lin et al. 1991;Yu and Wolfner 2002). The molecular mechanism of this gene is unknown. YA is present only in oocytes and early embryos (at the egg to embryo transition) and in no other developmental stage or adult tissue. Its function is essential for the male and female genomes to undergo their first cell cycle together. EMS mutagenesis screens have yielded two apparent null mutants, Ya^2 and Ya^{76}. Ya^2 is a deletion of the last 232 amino acids of YA, and Ya^{76} is a point mutation of Serine 226 to phenylalanine (see diagram in Chapter 3) (Liu et al. 1995). Drosophila embryos from homozygous Ya^2 mutant females (referred to as Ya^2 embryos) have abnormally condensed chromatin, and different haploid female meiotic products can have different condensation states from one another and from the male pronucleus in these embryos (Lin et al. 1991; Liu et al. 1995). YA protein is present in oocytes and embryos throughout the syncytial divisions, but is not present in later stages of embryo development or in larvae or adults (K. Song, thesis). Several observations demonstrate that YA function is only necessary for early embryonic mitosis. Homozygous Ya^2 males are normal, and females are as well, except that they are sterile because all their embryos arrest as described above. In addition, ectopic expression of YA does not have an effect on the cell cycle of post-embryonic tissues (Lopez et al. 1994).

YA localizes to the nucleoplasm and nuclear lamina of activated egg and embryo nuclei (Lopez et al. 1997), where it can interact with lamin Dm0. This protein-protein interaction has been demonstrated by yeast-two-hybrid (Goldberg et al. 1998) and both immunofluorescence and immuno- electron-microscopy colocalization (Lopez et al. 1997). YA can bind to chromatin, specifically to both DNA and Histone H2B, based on *in vitro* biochemical assays and immunofluorescence colocalization when ectopically expressed in cells containing polytene chromosomes (Lopez et al.

1997; Yu and Wolfner 2002). The *Ya*^{2} arrest phenotype of failure to progress through the first mitosis, and YA's presence in the nucleus, interacting with chromatin and the nuclear lamina, lead to the hypothesis that *Ya* might have a role in regulation of the first mitotic cell cycle. Genetic epistasis data with the *giant nuclei* (*gnu*) gene indicates that YA is necessary for the multiple rounds of DNA replication in that mutant. As described above, *gnu* mutant embryos undergo repeated DNA replication without mitosis, producing a small number of giant polyploid nuclei per embryo (Freeman et al. 1987). The *Gnu* null mutant in combination with either *Ya* null mutant shows a *Ya*-like phenotype, in which the nuclei are abnormally condensed and not giant (Liu, Lopez, et al. 1997). It is possible that the first S phase is completed in these double mutants, but the subsequent rounds of embryonic mitosis clearly do not occur. Fluorescence in situ hybridization (FISH) experiments done by J. Lopez to genotype *Ya* mutant nuclei have not completely clarified the ploidy of the nuclei (J. Lopez, PhD thesis, 1996). Even in wild type fertilized embryos, when S phase had presumably occurred in some fraction of the embryos observed, individual sister chromatids could not be distinguished from each other when the chromatids were not separated from each other.

The phosphorylation state of YA is developmentally regulated: like GNU (Renault et al. 2003) it is dephosphorylated upon egg activation. YA's dephosphorylation correlates with a change in its subcellular localization (Yu et al. 1999). YA is hyperphosphorylated in oocytes. In this state it is excluded from nuclei. This is hypothesized to be because phospho-YA binds preferentially to the cytoplasmic protein P0 and to other cytoplasmic proteins in a 20S complex (Yu et al. 2002). It has been proposed that YA is excluded from the meiotic nucleus of the oocyte, but upon activation it loses some phosphorylation(s), making it less tightly bound to the cytoplasmic retention complex. This allows YA to enter the nucleus, localizing to the

nuclear lamina and nucleoplasm as described above. Its nuclear entry and localization are probably mediated both by YA's carboxy-terminal nuclear localization sequences (NLS) and by YA's lamin binding (Mani et al. 2003). YA function could be regulated by sequestering the protein in the cytoplasm before its appropriate time of action, and then localizing it to the nucleus upon egg activation, with dephosphorylation providing the switch that allows the change in localization. This model of YA localization controlled by phosphorylation is supported by the fact that *in vitro*- phosphorylated YA binds better to P0 than non-phosphorylated YA (Yu et al. 2002). Bioinformatic analysis of the amino acid sequence of YA has identified putative phosphorylation sites for MAPK, CDK, GSK3, and other kinases. MAPKs (ERK, JNK, and p38) but not CDK phosphorylates YA in vitro (Yu et al. 2002 and J. Cui unpublished results). Because YA is not required for the events of egg activation tested in Chapter 5, and because its change in phosphorylation occurs upon egg activation, YA phosphorylation state can be assayed as an event that occurs downstream of egg activation.

1.5 Dissertation Outline

Changes in phosphorylation state are likely to be important for regulating multiple aspects of the oocyte to embryo transition. I have showed that MAP kinases are dephosphorylated upon egg activation. In addition, the egg activation genes *cort*, *sra,* and *prg* are upstream of YA dephosphorylation upon egg activation, and that *cort* and *sra* are also upstream of GNU dephosphorylation. I identified the phosphorylation sites on YA from oocytes and embryos by mass spectrometry. In oocytes, YA and GNU mRNA polyadenylation and protein levels are regulated by *wispy*, which also regulates ERK and JNK phosphorylation in oocytes, probably through *Mos*. I also clarified the time of the cell cycle when YA is necessary; YA acts after egg activation to regulate the entry into mitosis after the first S phase.

To examine the regulation of MAPKs during Drosophila egg activation and early embryogenesis, in Chapter Two I quantified the levels of phosphorylated (active) forms of ERK, p38, and JNK by western blotting with antibodies specific to the phospho- forms of these kinases. Levels of phospho-ERK, phospho-p38, and phospho-JNK are high in Drosophila oocytes. I found that upon egg activation, levels of all these phospho- (active) forms of MAPKs decrease. Fertilization is not required for this decrease, consistent with the independence of egg activation from fertilization in Drosophila. The decrease in levels of phospho-MAPK occurs normally in embryos laid by sterile females mutant in the egg activation genes *cortex*, *sarah*, and *prage*. The decrease in MAPK activity could be an intermediate step in the pathway leading from the calcium signal that initiates egg activation to the downstream events of activation.

In Chapter Three I test the relationships between known egg activation genes, selected kinases and phosphatases, and phospho-proteins regulated upon egg activation. I tested whether YA or GNU dephosphorylation required any of three genes known to be involved in egg activation (*cortex, sarah*, and *prage*), and also tested the requirement for separate MAPK activities (ERK vs. JNK and p38) in the dephosphorylation of YA and GNU. All three egg activation genes tested are upstream of YA dephosphorylation, but only *cort* and *sra* (not *prg*) are upstream of GNU dephosphorylation. Mass spectrometry analysis shows that YA is phosphorylated on two MAPK target sites and seven other amino acid residues in both oocytes and embryos, and on two oocyte-specific sites. Mutation of the activating kinases of one (ERK) or two (p38 and JNK) MAPKs does not have an evident effect on YA phosphorylation in oocytes, suggesting redundant functions. Although MAPKs and PP1 phosphatases are known to regulate GNU phosphorylation, neither inhibition of PP1 phosphatases by Nipp and Inhibitor-2 nor knockout of MKP3 in the germline

hinders GNU dephosphorylation upon egg activation, nor does the presence of constitutively active ERK.

In Chapter Four I show that ERK, JNK, YA, and GNU are regulated in oocytes by the *wispy* poly(A) polymerase. Levels of the phospho- (active) forms of ERK, MEK, and JNK are reduced in *wisp* mutant oocytes, which may be attributable to the lack of polyadenylation of *Mos* (a MAPKKK) mRNA. Total protein levels of YA and GNU are low in wildtype oocytes prior to maturation, and increase greatly upon maturation, which correlates with *wisp*-dependent polyadenylation of *Ya* and *gnu* mRNAs. Total protein levels of YA and GNU are low in *wisp* mutant mature oocytes, and the protein present at low levels is dephosphorylated. The normal increase upon oocyte maturation in YA and GNU levels (present in their phosphorylated forms), could provide sufficient levels of these proteins to perform their functions immediately after egg activation.

Chapter Five contains my contribution to showing that egg activation occurs normally in *Ya* mutant eggs and embryos. ERK and GNU dephosphorylation and SMAUG translation occur in YA-deficient embryos upon egg activation. Furthermore, after egg activation, S phase occurs in the absence of YA function, which indicates that the nuclei arrested prior to the first mitosis in mutant embryos have undergone one round of DNA replication before condensing their chromatin and arresting without entering the gonomeric mitosis.

In Appendix A, I compared YA amino acid sequence conservation across Drosophila and assayed for its presence in other insects, finding possible orthologs in other dipterans, but not from other orders. Based on sequence similarity to a conserved region of YA's sequence and mutant phenotype similarity, I identified the mouse gene *zar1* as a candidate functional homolog to *Ya*, but found that neither a *Ya* homolog from a distantly related Drosophila species nor *zar1* can rescue a *Ya* null

mutant, although YA from closely related Drosophila species can. Appendix B contains the results of a yeast two hybrid assay for nuclear envelope protein interactions with YA and other nuclear lamina proteins. In Appendix C I show that despite the egg-to-egg variation in rates of in vitro egg activation, there is a a trend towards ERK dephosphorylation occurring upon *in vitro* egg activation, and a trend towards an abnormal increase in p38 phosphorylation upon *in vitro* egg activation. Appendix D discusses the technical difficulties in detecting BrdU incorporation into individual meiotic products of Drosophila embryos. Appendix E contains the proteins identified by mass spectrometry as putative YA interactors because they co-purify with FLAG-YA.

CHAPTER TWO

MODULATION OF MAPK ACTIVITIES DURING EGG ACTIVATION IN DROSOPHILA[1]

2.1 Introduction

Egg activation is the developmental process that permits the transition of a mature egg to a cell that is capable of initiating embryonic development (Ducibella et al. 2006; Heifetz et al. 2001). Changes in the cell cycle occur during egg activation. Meiosis, which had been arrested in mature oocytes, resumes and completes; moreover, the activated oocyte becomes capable of initiating mitotic divisions after creation of the zygotic nucleus. In Drosophila, rearrangement of the sperm nucleus into a male pronucleus also depends on egg activation (Horner et al. 2006). Upon egg activation in Drosophila, as in many other animals, maternal RNAs and proteins that were pre-loaded into the mature oocyte must be properly localized and/or translated in order to direct the rapid mitotic divisions, patterning, and morphogenesis of the early embryo. This localization is accomplished through the degradation of certain maternal RNAs (Tadros et al. 2003) and the translation of others (Macdonald et al. 1986). In addition, activation causes cytoskeletal changes (Brunet et al. 2005; Theurkauf et al. 1992) as well as changes in the egg coverings to prevent polyspermy and to protect the egg from environmental damage (Heifetz et al. 2001; LeMosy et al. 2000).

Although egg activation is universal among metazoans, the mechanisms by which this process is initially triggered differ among organisms. In vertebrates and many marine invertebrates, the fertilizing sperm causes a rise in calcium levels within

1 This chapter is published as: Modulation of MAPK Activities During Egg Activation in Drosophila. Sackton KL, Buehner NA and Wolfner MF (2007) Fly 1(4):222-227. N. Buehner made figure 2.1B, and did some biological replicates of the experiments in figures 2.1A , 2.1B, and 2.2).

the egg; the calcium, in turn, initiates the events of activation (Ducibella et al. 2006). However, in the four insects examined to date (Drosophila and three wasps), fertilization is not needed for egg activation; instead, activation appears to be triggered by stimuli imparted to the ovulated egg as it passes through the reproductive tract (Heifetz et al. 2001; King et al. 1970; von Borstel 1957; Went et al. 1974). Recent studies have shown that, despite this difference in initial trigger, many of the downstream activation events in Drosophila including a requirement for calcium signaling (Horner et al. 2006; Takeo et al. 2006) are analogous to those in other animals. Drosophila can thus serve as a model in which to identify the genes that regulate activation.

Several maternal effect lethal mutations in Drosophila cause defects in specific aspects of egg activation. An example is *cortex*, which encodes a homolog of the Anaphase Promoting Complex/Cyclosome (APC/C) specificity factor, Cdc20 (Chu et al. 2001; Swan et al. 2007). Eggs laid by *cortex* mutant mothers arrest in metaphase of meiosis II, and have defects in mRNA translation and degradation (Lieberfarb et al. 1996; Page et al. 1996). Another conserved gene is *sarah,* encoding the Drosophila calcipressin, a regulator of the calcium and calmodulin-dependent phosphatase, calcineurin. Eggs laid by *sarah* mutant mothers arrest in anaphase of meiosis I and have defects in mRNA polyadenylation and translation, and in formation of the male pronucleus (Horner et al. 2006; Takeo et al. 2006). *Prage* mutants lay eggs that are defective in mRNA degradation and completion of meiosis (Tadros et al. 2003). Each activation gene discovered to date is necessary for some, but not all, aspects of egg activation (Horner et al. 2006; Takeo et al. 2006; Tadros et al. 2003; Page et al. 1996; Lieberfarb et al. 1996).

For the events of activation to occur rapidly and coordinately, the calcium signal feeds into several signaling pathways, including phosphorylation cascades in

vertebrates (Ducibella et al. 2006). However, much is still unknown about signal transduction during egg activation. One phosphorylation cascade characterized in several vertebrate and marine-invertebrate systems involves mitogen-activated protein kinases (MAP kinases; MAPKs) (Ducibella et al. 2006; Fan et al. 2004). In vertebrate egg activation, the Mos-MEK-MAPK-Rsk pathway establishes meiosis arrest in mature oocytes through multiple Rsk kinase targets (Maller et al. 2002; Nishiyama et al. 2007). After egg activation, Mos is degraded and MAPK activity drops (Inoue et al. 2007). Although Drosophila Mos is present in oocytes prior to ovulation, it is not necessary for cell cycle changes at egg activation, since *Dmos* null mutants complete meiosis normally despite decreased levels of ERK and MEK in mutant oocytes (Ivanovska et al. 2004). However, this finding does not eliminate the possibility of a role for modulation of MAPK activities during egg activation in Drosophila, given the numerous phenomena that occur during this time. Two Drosophila proteins that are established or potential MAPK targets, YA and GNU, become dephosphorylated during activation (Renault et al. 2003; Yu et al. 1999; Yu et al. 2002). These proteins' dephosphorylation suggests that MAPK activity levels might drop during activation in Drosophila, analogous to what has been reported in several marine invertebrates and vertebrates.

The Drosophila genome has representatives of each of the three MAPK subfamilies found in other animals: one extracellular signal-related kinase [ERK; *rolled* (*rl)*], one Jun N-terminal kinase [JNK; *basket* (*bsk*)], and p38 (encoded by the two genes *Mpk2* and *p38b*). MAPKs are activated by phosphorylation, with each MAPK targeted by a separate MAPKK that carries out this phosphorylation. All three Drosophila MAPKs have known functions in oocytes. ERK plays a role in dorso-ventral patterning in response to gurken (Mantrova et al. 1998), p38 is necessary for anterior-posterior axis positioning of oskar mRNA (Suzanne et al. 1999) and, late in

oogenesis, JNK participates in the formation of the dorsal appendages (Suzanne et al. 2001). Although antibodies are not available specifically against Drosophila MAPKs, anti-(rat)-ERK recognizes Drosophila ERK and antibodies prepared against the active (phosphorylated) forms of mammalian MAPKs recognize the three phospho-MAPK proteins in Drosophila oocytes and embryos (for examples see (Gabay et al. 1997; Ivanovska et al. 2004) and Materials & Methods). To determine if MAPK activities are modulated during Drosophila activation as they are in other organisms, we therefore used these antibodies to assess the phosphorylation state of MAPKs during egg activation. We find that mature late-stage Drosophila oocytes have high levels of the phospho- forms of all three MAPKs. However, levels of phospho-MAPK drop dramatically upon egg activation in a manner independent of fertilization. A decrease in MAPK activity corresponding to the decrease in phospho-MAPK could result in dephosphorylation of MAPK targets. This suggests that, like the situation in vertebrates and marine invertebrates, phosphorylation cascades play a key role in the signal transduction of Drosophila egg activation. Further, we found that levels of phospho-MAPK drop normally in activated eggs of *cortex*, *sarah*, and *prage* mutants, indicating that the corresponding gene products function downstream, or independently, of the regulation of MAPK activity.

2.2 Materials and Methods

Flies, oocytes and embryos:

Drosophila stocks were raised on standard yeast-glucose media at room temperature on a 12:12 light:dark cycle. Female flies collected as virgins were aged to 3-6 days old before use. OregonR P2 (Allis et al. 1977), Canton S, and w^{1118} were used as wild type controls. Other stocks used were: tud^{1} *bw sp* /CyO (FBal0017244) to make spermless males (Boswell et al. 1985), *wy* LF^{133} FRT^{101} which carries a *D-Sor*

mutant (FBal0042752) on the FRT chromosome (Janody et al. 2000), *w*; ovo^{D1}* FRT(*w^{hs}*)/*st^{1} betaTub85D^{D} ss^{1} e^{s}*/TM3, *sb^{1}* (FBst0002139), and *y w hep lic* FRT^{101}/*y w ovo^{D1}* FRT^{101} (Fbab0028741) (Suzanne et al. 1999), to make germline clones, *sra^{687}*/TM3 (FBal0175443), and *Df(3R)sbd45, mwh^{1} e^{1}*/TM6 (FBst0003678), to make *sra^{687}* hemizygotes (Horner et al. 2006), *cort^{QW55} cn^{1}bw^{1}*/CyO, *l(2)DTS^{5131}* (Fbst0004974) (Page et al. 1996), and *Df(2L)BSC9, w^{+mC}*/SM6a (FBst0006454) to make *cort^{QW55}* hemizygotes, *prg^{16A}*/FM6, and *prg^{32}*/FM6 (FBal0151626)(Tadros et al. 2003). Germline clones were generated by heat-shock-induced site-specific recombination (Chou et al. 1992; Janody et al. 2000; Suzanne et al. 1999).

For examination of proteins from mature unactivated oocytes, stage 13-14 oocytes from 3-5 day old flies were dissected in phosphate buffered saline (137 mM NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 0.1% Triton X-100). Buffer was removed and the oocytes were flash-frozen in liquid nitrogen. For examination of proteins from activated but unfertilized eggs, we mated female flies of the wild type strain to spermless males who were produced by mating *tud^{1} bw sp* virgin females to Canton S or OregonR P2 males. The sons of this cross make and transfer seminal fluid proteins to induce high levels of egg production and ovulation in their mates, but because they do not produce sperm the eggs laid by their mates are unfertilized (Boswell et al. 1985; Kalb et al. 1993). Unfertilized eggs were collected for 1 hour on grape-juice agar plates. For examination of proteins from early embryos, we mated wild type females to wild type males and collected the embryos on grape-juice agar plates, for the time periods noted in the figures or text. Activated eggs and embryos were dechorionated in 50% bleach for 2 minutes, washed in egg wash buffer (Karr et al. 1986) and flash-frozen in liquid nitrogen.

Protein extraction and western blots:

Frozen samples described above were homogenized in protease inhibiting

homogenization buffer (PIHB; (Monsma et al. 1988)) with the addition of two phosphatase inhibitors, 20mM sodium fluoride and 10mM b-glycerophosphate. Total protein was quantified by Bio-Rad Protein Assay (#500-0006, Bio-Rad Laboratories, CA). Samples were prepared for gel electrophoresis by boiling in sample-buffer (Monsma et al. 1988), and 20-60 micrograms of protein were loaded into each lane of a 10.6% SDS polyacrylamide gel. Western blots were prepared as previously described (Lung et al. 1999; Monsma et al. 1988). Anti- (total) ERK, anti-phospho-ERK, anti-phospho-p38 or anti-phospho-JNK (Cell Signalling Technology, MA) were used at dilutions of 1:1000, 1:1000, 1:500, and 1:250, respectively, according to manufacturer's recommendations. We used anti- (total) ERK staining as a loading control. Levels of total ERK do not change between the samples as compared to a second loading control, alpha-tubulin (data not shown). Western blots of activation mutants focused on active ERK for technical reasons (anti-phospho-ERK antibody is more robust than anti-phospho-JNK antibody, and we have observed variation in levels of phospho-p38 (in *in vitro* samples) that is attributable to extraneous experimental factors ie. buffers and temperature changes during sample preparation).

After incubation with primary antibody overnight at 4°C, blots were probed with goat-anti-rabbit HRP secondary antibodies (Jackson Immunochemicals, PA) at a 1:2000 dilution, according to manufacturer's recommendation. Signals were detected with ECL-Plus (GE Healthcare, United Kingdom), on a Storm™ imager (GE Healthcare, United Kingdom) and quantified with ImageQuant v1.2 software (GE Healthcare, United Kingdom). Each result shown was repeated in at least three independent experiments. To control for genetic background effects or blot-to-blot variation in loading, processing or film-exposure, signals each mutant's samples were compared to those for sibling controls that were extracted in parallel and run on the same gel (Figure 2.2).

2.3 Results

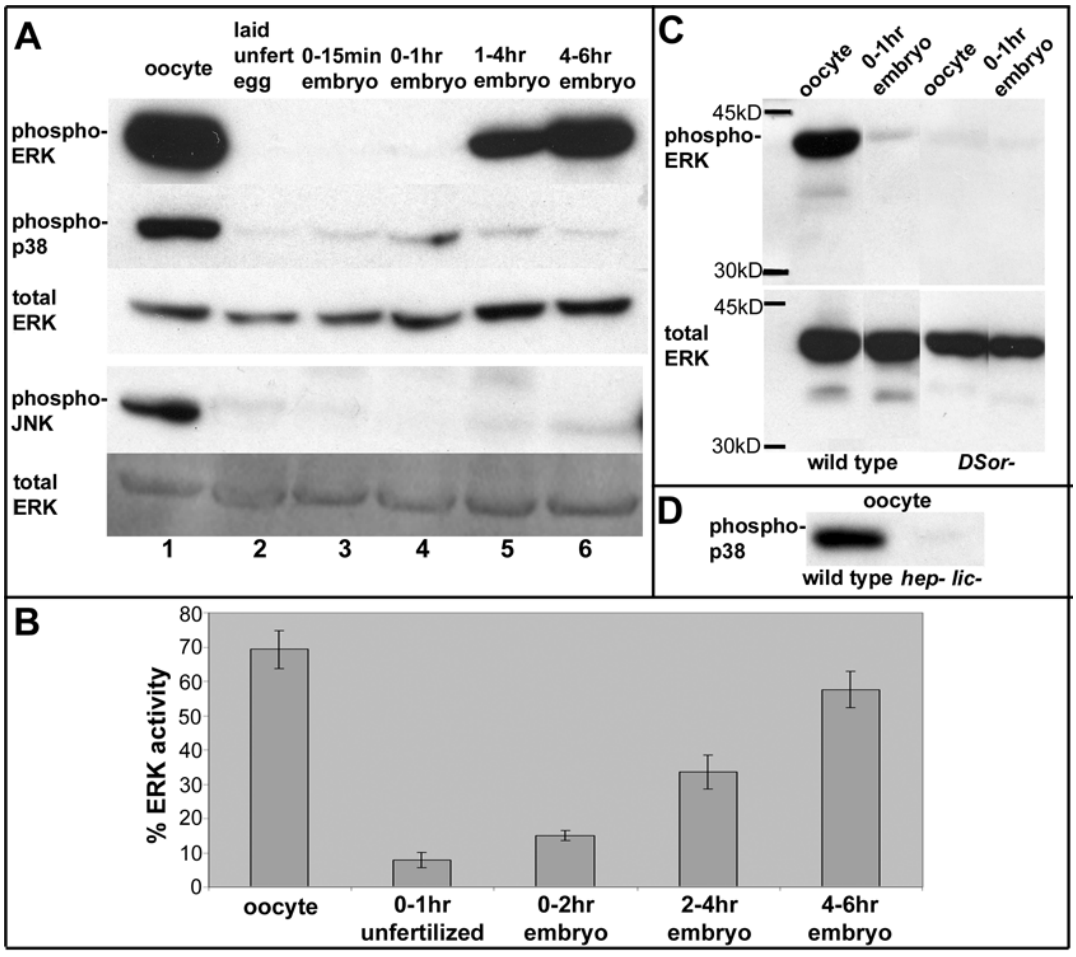
Phosphorylated forms of all three MAPKs are detected in oocytes:

Phospho-ERK, phospho-p38, and phospho-JNK are all present in mature stage 13-14 oocytes, which are arrested at metaphase of meiosis I (Figure 2.1A, lane 1). We verified the specificity of the phospho-ERK and phospho-p38 antibodies by comparing wild type oocytes with germline clones lacking either the ERK kinase (Dsor) or both the p38 kinase (Lic) and the JNK kinase (Hep) (Suzanne et al. 1999; Janody et al. 2000); in such oocytes we did not detect phospho-MAPK signal in the absence of the appropriate MAPKK (Figure 2.1 C & D). We detected phospho-ERK in early (stage 1-10) oocytes at levels comparable to older (stage 11-14) oocytes (data not shown). Having confirmed that we can detect MAP kinases by this method in oocytes, and given their potential involvement in egg activation, we examined MAPK and active (phospho-) MAPK levels in activated eggs and embryos.

Levels of phospho-MAPKs drop during the egg to embryo transition:

Extracts were prepared from mature oocytes and from fertilized eggs. Proteins were extracted from embryo collections of various lengths, and their levels of phospho-ERK, phospho-p38 and phospho-JNK were assayed by western blotting with the phospho-specific antibodies described above. Levels of all three phospho-MAPKs decrease dramatically and rapidly during the egg-to-embryo transition (Figure 2.1A, compare lanes 1 & 3). Average levels of phospho-ERK remain low during the syncytial mitotic divisions (which occur during 0-2 hours after egg laying), then increase after cellularization (Figure 2.1A, compare lanes 3 & 4 to 5 & 6, Figure 2.1B compare 3rd bar to 4th and 5th bar). Phospho-p38 and phospho-JNK levels remain low or undetectable throughout the first six hours of embryo development (Figure 2.1A, lanes 3-6).

Figure 2.1 Levels of phospho-MAPKs during the egg-to-embryo transition (A) Western blot showing levels of active (phospho-) forms of ERK, p38, and JNK, respectively, and ERK protein levels (total ERK), in mature unactivated (ovarian) oocytes, in unfertilized eggs, and in early embryos of the indicated ages. Equivalence of protein loading is verified by the total-ERK staining. Phospho-ERK, phospho-p38, and total ERK from the same gel are shown, and a pair of phospho-JNK and total ERK from another gel. Levels of active ERK, p38, and JNK decrease at the oocyte to activated egg or embryo transition (compare lane 1 to lanes 2 and 3). Levels of active MAPK are low (and comparable) in unfertilized eggs (lane 2) and early-stage (lanes 3 and 4) embryos, although levels of active ERK rise after the syncytial stage of embryogenesis (lane 6). (B) Quantitation of phospho-ERK levels relative to total ERK activity. Scans of western blots like that shown in panel A were quantified by Image Quant software analysis and levels of phospho-ERK were normalized to total ERK. Results shown are the average of five or more experiments each. During the transition from oocyte to activated egg or embryo, levels of phospho-ERK decrease roughly 4-fold, both for fertilized (3rd bar) and unfertilized (2nd bar) eggs, indicating that the drop in levels of phospho-ERK coincides with activation. (C) Phospho-ERK is not detected in oocytes of *DSor*- germline clones (compare to OregonR P2 (wild type) oocytes) while total ERK loading control is detected in all oocytes and embryos. Lanes shown are non-adjacent lanes from the same blot. The MAPKs run between the 45kD and the 30kD molecular weight markers, closer to the 45kD marker. (D) Phospho-p38 is not detected in oocytes of *hep,lic* germline clones (compare to OregonR P2 (wild type) oocytes).


A
oocyte
laid unfert egg
0-15min embryo
0-1hr embryo
1-4hr embryo
4-6hr embryo
phospho-ERK
phospho-p38
total ERK
phospho-JNK
total ERK
1
2
3
4
5
6
C
oocyte
0-1hr embryo
oocyte
0-1hr embryo
45kD
phospho-ERK
30kD
45kD
total ERK
30kD
wild type
DSor-
D
oocyte
phospho-p38
wild type
hep-
lic-
B
% ERK activity
80
70
60
50
40
30
20
10
0
oocyte
0-1hr unfertilized
0-2hr embryo
2-4hr embryo
4-6hr embryo

Decrease in levels of phospho-MAPK during egg activation is independent of fertilization:

We can distinguish effects of activation *per se* from effects of fertilization because Drosophila females lay activated, unfertilized eggs. In contrast, in organisms whose *in vivo* activation trigger is fertilization, regulation of changes in MAPK activity during egg activation can only be studied independent of fertilization using *in vitro* systems. To test whether the drop in MAPK activity is dependent on fertilization in Drosophila, we collected eggs laid by females mated to spermless males. These eggs are activated *in vivo* but not fertilized, and are completing or have completed meiosis. Levels of phospho-ERK in laid unfertilized eggs are similar to the low levels seen in early embryos (Figure 2.1A lanes 2 & 4, Figure 1B 2nd & 3rd bars). The levels of the other two phospho-MAPKs also decrease upon egg activation independent of fertilization (Figure 2.1A compare lane 1 to lanes 2 & 3). Thus egg activation, not fertilization, triggers the decrease in phosphorylation of MAPKs.

The decrease in levels of phospho-ERK occurs normally in three activation mutants:

We examined the decrease in ERK activity upon egg activation in three mutants (*cortex*, *sarah*, and *prage*) that affect various subsets of the events of egg activation (see above). In oocytes from *cortex*, *sarah*, and *prage* mutant females, levels of phospho-ERK are high, as in genotype-matched controls (Figure 2.2). Upon activation in embryos laid by *cortex*, *sarah*, and *prage* females, the levels of phospho-ERK decrease to levels similar to those seen in controls (Figure 2.2).

3.4 Discussion

MAPK signaling plays important but incompletely characterized roles in oocyte maturation, egg activation, and early embryogenesis in animals. We have observed that levels of phospho-ERK, phospho-p38 and phospho-JNK are much

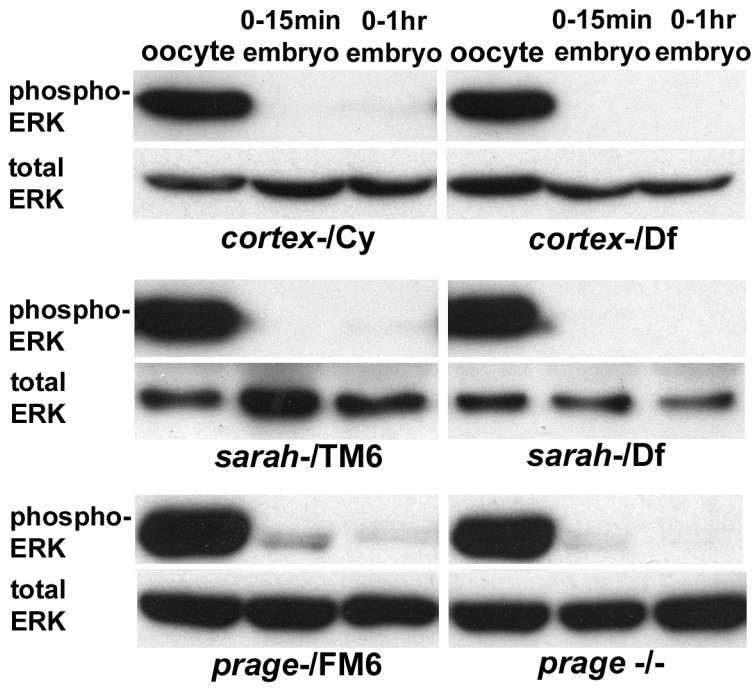


Figure 2.2: ERK phosphorylation decreases normally upon activation in mutants

Western blot of phospho-ERK and total ERK from mature oocytes and 0-2hr embryos from homozygous or hemizygous mutant females or from their sibling controls (mutant/balancer). Maternal genotype is indicated below the corresponding western blots. In the activation mutants *cortex* (rows 1&2), *sarah* (rows 3&4) and *prage* (rows 5&6), levels of phospho-ERK decrease at the oocyte to embryo transition.

higher in mature Drosophila oocytes than in activated eggs or early embryos. Similar changes in MAPK activity have been reported in the egg-to-embryo transition in several other non-insect animals (Ducibella et al. 2006; Whitaker 1996). However, unlike these other species, insect eggs activate independent of fertilization. Our results show that in Drosophila levels of phospho-MAPK decrease as a consequence of activation and not as a response to fertilization. Decreased MAPK activity (loss of phospho-MAPK) upon activation could cause rapid dephosphorylation of MAPK targets. Therefore our findings suggest that MAPKs could be one of the links between the trigger of activation and subsequent activation events.

Previous studies have described the roles of ERK, p38, and JNK in Drosophila oocyte patterning. Germline clone analyses that remove the maternal contribution of a MAPKK (disrupting *Dsor*, *lic*, or *hep*, respectively) cause distinct patterning defects in oocytes and embryos (Suzanne et al. 1999, 2001; Janody et al. 2000). Since the first reported embryo defects from these MAPKK mutant germline clones are seen in segmentation and other aspects of patterning during cellularization, meiosis apparently completes upon their activation, and embryonic mitosis initiates (our unpublished observations and (Janody et al. 2000)). As we have shown, all three MAPKs become dephosphorylated (inactivated) upon activation. In contrast, germline clones only have inactivated some MAPKs, for example, ERK alone (*Dsor*) (Janody et al. 2000) or p38 and JNK (*hep,lic*) (Suzanne et al. 1999, 2001) either singly or together. Redundancy between the MAPKs may explain why early defects, such as premature activation or defects in syncytial mitosis, have not been observed in the embryos that lack activity of only one or two MAPKs. In some cell types of Drosophila and other organisms, MAPKs have been shown to regulate cell cycle progression (Inoue et al. 2007; Johnson et al. 2002). Our detection of low levels of phospho-MAPKs in 0-1 and 0-2 -hour-old embryos might reflect a presence, or transient presence, of active

MAPK during the syncytial mitotic divisions. High levels of ERK activity over the whole embryo are not restored until after cellularization (Figure 1, lanes 5 & 6).

Modulation of MAPK activity is expected to affect the phosphorylation state of MAPK targets. Global changes in protein phosphorylation are ideally suited to effect rapidly the multiple changes that occur during egg activation. Indeed, a proteomic screen in sea urchins has detected a number of proteins that are dephosphorylated upon egg activation (Roux et al. 2006). Targets of MAPK phosphorylation could be involved in several different events of activation. The best characterized of these events is the resumption and completion of meiosis: during Xenopus egg maturation and activation the Mos-MEK-MAPK-Rsk pathway directly regulates the cell cycle through MPF (Maller et al. 2002). The only two proteins in Drosophila known to be dephosphorylated upon activation, GNU and YA, are MAPK substrates but are probably not involved in the release of meiotic arrest, because they have no obvious effects before activation (Renault et al. 2003; Yu et al. 1999; Yu et al. 2002; Zhang et al. 2004 and Chapter 5). Further genetic and proteomic studies in Drosophila should identify regulators of MAPK activity and link MAPK substrates to the biochemical and cellular events of egg activation.

Our results show that neither *cortex*, *sarah*, or *prage* are upstream regulators of the decrease in ERK phosphorylation. Analogous to eggshell hardening (Tadros et al. 2003), the decrease in ERK phosphorylation is an aspect of activation that occurs normally in these three mutants. These genes play critical roles in activation that could be downstream of the decreased MAPK phosphorylation or independent of the MAPKs. No role for APC-mediated proteolysis in regulating MAPK activity has previously been reported, consistent with our results presented here for *cortex* mutants. *Sarah* regulates a phosphatase (Horner et al. 2006) that could potentially have had a role in regulating MAPK activity. However, we have shown that calcipressin (*sra*)

function is not necessary for the decrease in ERK activity. We will be particularly interested in establishing whether the decrease in ERK phosphorylation upon activation regulates the Cortex, Sarah or Prage proteins' activities. Determining the relationship between MAPK activity, various events of egg activation, and genes known to function at this time, using Drosophila as a model system, will contribute greatly to our understanding of the critical developmental transition from oocyte to embryo.

CHAPTER THREE

PHOSPHO-SIGNALLING DURING EGG ACTIVATION

3.1 Introduction

Phosphorylation can regulate protein activity by a number of mechanisms, and it can be both activating or inhibitory. For example, the presence or absence of the phosphate group can alter an enzyme's structure thereby regulating its enzymatic activity [e.g. MAPK (Bott et al. 1994)]. Phosphorylation can also be required for or hinder protein-protein interactions [e.g.14-3-3 proteins (Mackintosh 2004)]. Egg activation quickly transforms a quiescent oocyte into a developing embryo, and so regulation of the proteins that bring about egg activation must be rapid. Phosphorylation is such a rapid regulatory mechanism.

Several lines of evidence suggest the importance of phosphorylation changes in egg activation. First, two Drosophila phosphoproteins known to act at the egg-to-embryo transition (but after egg activation), YA and GNU, are dephosphorylated upon egg activation (Renault et al. 2003; Yu et al. 1999). Second, levels of phospho-MAPKs decrease upon egg activation in Drosophila, Xenopus, mice, and some marine invertebrates (Ferrell et al. 1991; Kubiak et al. 1993; Sackton et al. 2007; Sanghera et al. 1991; Shibuya et al. 1992)(Chapter 2). This in itself is a change in phosphorylation (MAPKs are phosphorylated to be active), and also has the potential to affect phosphorylation levels of any MAPK protein targets present in mature oocytes, which may therefore become less phosphorylated during egg activation. Third, proteomic screens in sea urchin (Roux et al. 2006; Roux et al. 2008) and Drosophila (V. Horner, A. Krauchunas, and M. Wolfner, unpublished results) have observed large numbers of proteins whose phosphorylation state changes upon egg activation (109 change in

Drosophila, comparable to ~100 in sea urchin, although this exact statistic has not been published), and relatively few have been identified. Some become phosphorylated or more phosphorylated (7 proteins identified in sea urchin), others are dephosphorylated either fully or partially (4 proteins identified in sea urchin). Identifying these phosphoproteins and determining their roles in egg activation are currently areas of active research. I expect that phosphoproteins will be part of the molecular pathways that connect calcium, the initial signal of egg activation, with egg activation genes and with the downstream events that characterize egg activation. Since the proteomic studies are still at a very early stage, I have focused my analysis on proteins previously known to change phosphorylation state upon egg activation; YA, GNU, and the MAPKs.

MAP Kinases

Knowing that levels of active (phospho-) MAPKs decrease upon egg activation in Drosophila as in other organisms (e.g. Xenopus, mice, pigs, perch), we sought connections between this change in kinase activity and other events of egg activation. In other organisms, MAPKs are involved in regulating meiotic progression downstream of *Mos* (Sagata et al. 1989; Madgwick et al. 2007), but in Drosophila, although ERK and MEK phosphorylation are *dmos*-dependent, meiotic progression is not *dmos*-dependent. *Dmos* null mutants lay eggs that can complete meiosis, and are fertile to near-normal levels (Ivanovska et al. 2004). However, other events of egg activation in Drosophila, such as YA or GNU dephosphorylation, may depend on the decrease of MAP kinase activity. MAPKs have roles both before and after egg activation in Drosophila. During oogenesis, the p38- (MAPK) activating kinase *lic* is required for anterior-posterior patterning (Suzanne et al. 1999), ERK is involved in dorso-ventral patterning (Mantrova et al. 1998), and the JNK-activating kinase *hep* is required in the follicle cells to form the dorsal appendages of the eggshell (Suzanne et

al. 2001). The elimination of any one of these kinases' function in the germline causes embryogenesis defects and arrest shortly after cellularization (Janody et al. 2000; Sackton et al. 2007; Suzanne et al. 1999), so in Drosophila early development, maternal MAPK activity is primarily required during oogenesis or for events after cellularization, or they have redundant functions before cellularization. If the inactivation of MAPKs in early embryos is important for the period of development after oogenesis and before cellularization, it could be because their kinase activity would prevent the necessary dephosphorylation of MAPK targets. Based on the hypothesis that YA and GNU are negatively regulated by phosphorylation in oocytes and function in their dephosphorylated forms immediately after egg activation but before cellularization, I tested whether elimination of either one or two of the MAP kinases' activity in the germline had an effect on YA or GNU phosphorylation in oocytes.

Other Kinases

Other kinases are present in oocytes and embryos, and their expression at this stage in development makes them candidate egg activation regulators. Kinases which have known functions in Drosophila, such as GSK3 (a component of the Wnt, Hedeghog, and Wingless patterning pathways (Jia et al. 2002)), could have other targets that mediate events of egg activation. Some kinases, such as CaMKII, have demonstrated roles in egg activation in other organisms. Specifically, CaMKII inhibits Emi1, an inhibitor of the APC, thus triggering cyclin B degradation in Xenopus (Rauh et al. 2005). The Emi1 homolog in Drosophila, Rca1, does not have a meiotic phenotype, but regulates cyclin A and has an arrest phenotype in embryonic cell cycle sixteen (Dong et al. 1997; Reimann et al. 2001). Polo kinase is also involved in the Emi pathway regulating meiotic progression in vertebrates (Schmidt et al. 2005), and *polo* null mutant Drosophila eggs have defects in meiosis and the first mitosis

(Riparbelli et al. 2000). Another possible role for these three kinases is in YA phosphorylation, since predictions of kinase consensus motifs suggest they may phosphorylate sites on YA that I have shown by mass spectrometry are phosphorylated (this chapter). These kinases are not directly part of my studies of kinases in egg activation, but the possible involvement of these or other kinases in egg activation is considered in the analysis of my results.

Phosphatases

To shift the equilibrium of a protein's phosphorylation state towards the dephosphorylated form, either kinase activity must decrease, phosphatase activity must increase, or both. Phosphatases are classified broadly by the amino acid residues they dephosphorylate, and the two sets of proteins known to be dephosphorylated upon egg activation would require two different sets of phosphatase. The first set of phosphoproteins, the MAPKs, are phosphorylated on the critical regulatory motif Thr-X-Tyr, and so can be inactivated by dephosphorylation by Ser/Thr phosphatases such as PP2A, or by protein tyrosine phosphatases (which include dual-specificity phosphatases) (Roux et al. 2004). Drosophila MAP Kinase Phosphatase 3 (MKP3) is a protein tyrosine phosphatase with demonstrated Tyr phosphatase activity and specificity for the dephosphorylation of ERK much more than JNK or p38 (Kim et al. 2002). Although MKP3 would not be expected to dephosphorylate YA, germline clones lacking MKP3 have a similar arrest phenotype to YA, with fewer than 5 nuclei present in the embryo (Kim et al. 2004). I further characterized this arrest and found that MKP3-deficient embryos can be fertilized and probably complete meiosis before they arrest, but the egg activation events tested occured normally in these embryos (see below). The second set of proteins dephosphorylated upon egg activation, YA and GNU, have serine and threonine phosphorylations, so they would be dephosphorylated by a Ser/Thr phosphatase such as PP1 or PP2A phosphatases. I have inhibited PP1

phosphatases in the germline by overexpression of the inhibitory proteins Nipp and Inhibitor-2 (Bennett et al. 2003), and observed decreased fertility due to reduced hatchability, as would be expected for a disruption of egg activation. However, individual events of egg activation such as GNU dephosphorylation are unaffected or have only mild and/or variable defects caused by the inhibition of the PP1 class of phosphatases. The serine/threonine phosphatase calcineurin is a likely regulator of egg activation because it is activated by calcium, and has been shown to regulate resumption of meiosis in Xenopus (Mochida et al. 2007). Our collaborators S. Takeo and T. Aigaki are currently studying calcineurin's role in Drosophila egg activation.

YA and GNU are MAPK substrates

In Chapter Two I showed that MAPK activity decreases during egg activation, making MAPKs a logical target to test for a role in YA/GNU phosphorylation change. Indeed, GNU and YA both have MAPK target amino acid motifs (Gonzalez et al. 1991; Jacobs et al. 1999) in their protein sequences. There are six serines/threonines in putative MAPK target amino acid motifs in GNU (Renault et al. 2003). Three of these residues occur within a single six amino acid sequence near the protein's amino terminus. There are also five predicted MAPK target amino acid motifs in YA (Yu et al. 1999), and YA can be phosphorylated by ERK in vitro (Yu et al. 2002). Studies of both proteins have been done using transgenic flies expressing mutant proteins with substitutions of Ser/Thr to Ala in consensus MAPK phosphorylation site(s) (Yu et al. 1999; Zheng et al. 2005). These transgenes were expressed in null mutant backgrounds so the only copy of the protein was the alanine-substitution mutant. Mutation of the three closely spaced MAPK target sites near the amino terminus of GNU (3S/T>A) results in a protein that no longer migrated like wildtype phosphorylated oocyte protein in transgenic oocyte extracts, but was more embryo-like in gel mobility. This 3S/T>A transgene caused a partially penetrant rescue of the

gnu null mutant fertility defect (Zheng et al. 2005). The rescue data are consistent with the developmental phospho-regulation of GNU, because it is dephosphorylated GNU that functions in the Pan Gu kinase complex to regulate early embryonic mitotic cycles (although the correlation does not mean that phospho-GNU in inactive). Indeed, phosphorylation of either GNU or YA in oocytes seems unlikely to contribute to their functions after egg activation, but is more likely a negative regulatory modification to prevent their function before egg activation, because they both act in embryos after activation, when they are less phosphorylated. This hypothesis is part of a model for developmental regulation of YA's subcellular localization (Yu et al. 2002). Because YA has higher affinity when phosphorylated for the cytoplasmic protein it interacts with in oocytes (P0), YA is hypothesized to be sequestered in a cytoplasmic complex in oocytes, and allowed to enter nuclei when it is dephosphorylated and released from the complex. Recombinant YA phosphorylated in vitro immunoprecipitates more recombinant P0 than non-phosphorylated recombinant YA.

Mutation of a single MAPK target site, T443 in YA (T443A), results in a protein that migrates faster in oocytes than wildtype oocyte YA, but not as fast as embryo YA (Yu et al. 1999). This suggests that T443 is only one of multiple sites phosphorylated on YA. This was confirmed by my mass spectrometry identification of phosphorylated sites as described later in this Chapter. The T443A mutation, or deletion of the five amino acids in the MAPK target amino acid motif, result in nonfunctional or barely functional (10-30% rescue) YA proteins, respectively (Liu et al. 1998). In addition, T443A can have dominant lethal effects on pupal development. Phosphorylation of T443 in embryos may be necessary for YA function based on the lack of robust rescue of the *Ya* null mutant fertility defect, despite the difficulty in detecting any difference in embryo YA electrophoretic mobility between wildtype and T443A proteins (Yu et al. 1999). Mutation of all five of YA's Ser/Thr in MAPK target

amino acid motifs (allA) results in oocyte allA-YA that migrates significantly faster than wildtype oocyte YA, and is also nonfunctional and dominant lethal in pupal development (N. Buehner and S. Mani, unpublished results). The dominant lethal phenotype is due to the T443A mutation, which has the same phenotype when mutated alone, so if other sites mutated in allA also contribute to YA function in embryos, their phenotypes would be masked by the T443A mutation's. Neither of the YA mutant constructs described affect whatever oocyte-specific YA phosphorylation is thought to be important in regulating subcellular localization, arguing that MAPK phosphorylations are not the regulators of subcellular localization. The significance of studies of MAPK site alanine-substitution mutants in YA and GNU is that because these mutations can alter the electrophoretic mobility of YA and GNU in oocytes, these proteins are shown to be phosphorylated by the active MAPKs present in oocytes (consistent with the bioinformatic predictions and in vitro phosphorylation assays), and can be further studied in relation to the change in MAPK activity upon egg activation. My mass spectrometry results (this chapter) confirm that YA is phosphorylated by MAPK, but also show that YA is a target of other kinases.

A gene involved in mediating the dephosphorylation of YA and GNU during egg activation could either promote the inactivation of a YA- or GNU-phosphorylating kinase, or increased activity of a YA- or GNU-dephosphorylating protein phosphatase. To create a complete model of the dephosphorylation of YA or GNU upon egg activation we need the following information. What connects calcium to the regulation of kinases and phosphatases? This is obvious for CamKII , calcineurin, and sarah, but what do they control? How are *cortex* (cdc20) or *prage* connected to calcium, and what phosphorylation events are mediated by *cort, prg,* or *sra*? Which kinases phosphorylate YA or GNU in oocytes? How many kinases each (one or several per protein) that act on GNU or YA could be regulated upon egg activation? Are the

phosphatases that dephosphorylate GNU or YA regulated upon egg activation? I have begun to address most of these questions directly or indirectly by the studies presented in this chapter. There is evidence for both YA and GNU being regulated by MAPKs. None of the three egg activation genes tested (*cortex*, *sarah*, and *prage*) are necessary for the decrease in phospho-ERK (Sackton et al. 2007). Therefore for one of these three activation genes to regulate GNU or YA dephosphorylation upstream of a kinase, GNU or YA must be phosphorylated by more than one kinase. That is, c*ortex*, *sarah* or *prage* could be required for the dephosphorylation of YA or GNU if the gene were upstream of the inactivation upon egg activation of another (non-MAPK) YA- or GNU-phosphorylating kinase. Alternatively, c*ortex*, *sarah* or *prage* could be required for the dephosphorylation of YA or GNU if the gene were upstream of an increase in YA- or GNU-dephosphorylating phosphatase activity (phosphatase regulation). My data show that c*ortex*, *sarah* and *prage* are upstream of the dephosphorylation of YA, but only c*ortex* and *sarah* are upstream of the dephosphorylation of GNU. Although *sra, cort,* and *prg* do not control MAPKs (Chapter 2), they do mediate phosphorylation of the MAPK targets YA and GNU.

3.2 Materials and Methods

Flies, oocytes and embryos:

Drosophila stocks were raised on standard yeast-glucose media at room temperature on a 12:12 light:dark cycle. Female flies collected as virgins were aged to 3-6 days old before use. OregonR P2 (Allis et al. 1977), Canton S, and w^{1118} were used as wild type controls. Other stocks used were: *wy* LF^{133} FRT^{101} which carries a *D-Sor* mutant (FBal0042752) on the FRT chromosome (Janody et al. 2000), *w*;* ovo^{D1} $FRT(w^{hs})/st^{1}$ $betaTub85D^{D}$ ss^{1} e^{s}/TM3, sb^{1} (FBst0002139), and *y w hep lic* FRT^{101}/*y w* ovo^{D1} FRT^{101} (Fbab0028741) (Suzanne et al. 1999), to make MAPKK germline clones,

FRT^{79D} DMKP3^{P1}/TM6B (gift of J. Chung, Korea AIST), *y w* hs-FLP;;*sb*/TM6B, w;;*ovo^{D1}* FRT^{79D} e^{s} /TM3*sb* (the latter two stocks were crossed to make males of the genotype *y w* hs-FLP/Y;; *ovo^{D1}* FRT^{79D}/*sb*) (Kim et al. 2004) to make MKP3 germline clones, *sra^{687}*/TM3 (FBal0175443), and *Df(3R)sbd45, mwh^{1} e^{1}*/TM6 (FBst0003678), to make *sra^{687}* hemizygotes (Horner et al. 2006), *cort^{QW55} cn^{1}bw^{1}*/CyO, *l(2)DTS^{5131}* (Fbst0004974) (Chu et al. 2001), and *Df(2L)BSC9, w^{+mC}*/SM6a (FBst0006454) to make *cort^{QW55}* hemizygotes, *prg^{16A}*/FM6, and *prg^{32}*/FM6 (Fbal0151626) (Tadros et al. 2003). The Gal4 driver lines *maternal tubulin-Gal4* (stock #7063) (Elliott et al. 2008) and *nanos-Gal4* (stock #4937) (Van Doren et al. 1998) are from Bloomington stock center. Germline clones were generated by heat-shock-induced site-specific recombination. For examination of proteins from oocytes, indicated stages of oocytes from 3-6 day old flies were dissected in phosphate buffered saline (137 mM NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 0.1% Triton X-100). Buffer was removed and the oocytes were flash-frozen in liquid nitrogen. For examination of proteins from early embryos, I mated females to wild type males and collected the embryos on grape-juice agar plates, for the time periods noted in the figures or text. Activated eggs and embryos were dechorionated in 50% bleach for 2 minutes, washed in egg wash buffer and flash-frozen in liquid nitrogen and stored at -80 degrees C.

Transgenic flies:

UASp-FLAG-YA and UASp-YA-FLAG were generated by cloning the *Ya* full-length cDNA coding sequence (using primers YAGWFwd 5'-**CACC**ATGTCGTTTTCCAATGTCCTAATC and YARev 5'-CTACTGGCGACGCATGCG and ORP2 oocyte cDNA template) into Gateway (Invitrogen, Carlsbad, CA) entry clone and subsequently (via LR reactions) into *P*-element transformation vectors which include the 3X-FLAG sequence either N-

terminal or C-terminal to the Gateway insertion site (gift of T. Murphy, Carnegie Institute of Washington, MD). UASp-Inhibitor 2, UASp-Nipp, and UASp-rl^{Sem} were generated by cloning full-length cDNA coding sequences into Gateway entry clones (using primers I2GWFwd 5'-**CACC**ATGCAGAACAATCCCAGCCC, I2Rev 5'-CTAGTTATTCGATGGCTCCAGGTC, NippGWFwd 5'-**CACC**ATGGCTAACAGCTACGACATAC and NippRev 5'-CACCATGCAGAACAATCCCAGCCC and ORP2 oocyte cDNA template) and then into untagged Gateway *P*-element transformation vectors (gift of T. Murphy, Carnegie Institute of Washington, MD). The *rl^{Sem}* allele was reconstructed via QuikChange site-directed mutagenesis (Stratagene, LaJolla, CA) of a Gateway entry clone containing the full-length cDNA coding sequence of *rl* (made using primers rlFWDgw 5'-CACCATGGAGGAATTTAATTCGAGCGG, rlREV 5'-CATTCTTAAGGCGCATTGTCTGG and ORP2 oocyte cDNA template) with the mutagenic primers (5' - GCAATATTATGATCCTGGAAATGAGCCTGTCGCTGAAGTG and 5' - CACTTCAGCGACAGGCTCATTTCCAGGATCATAATATTGC) to create the D334N amino acid substitution. For expression controlled by the *bcd* 3'UTR, the entire construct *P{w$^+$ gnuGFP-bcd3'UTR}* (Zhang et al. 2004) which is in a pCasPer4 vector, was moved into a pUC19 vector by digests with XbaI and EcoR1 and ligation. This plasmid was mutagenized by site-directed mutagenesis (Stratagene) to create a KpnI site near the start of the *gnu* CDS, and to change the BamHI site at the end of the GFP sequence to a Kpn1 site. The *P{w$^+$ gnuGFP-bcd3'UTR}* construct with new restriction enzyme recognition sequences was returned to the pCasPer4 transformation vector. KpnI digest of this vector removed the GNU-GFP coding sequence, and the *rl^{Sem}* coding sequence PCR product from KpnI-site-containing primers was inserted between the *gnu* 5'UTR and the *bcd* 3'UTR, creating *P{w$^+$ gnu5'UTR-rl^{Sem}-*

bcd3'UTR}. Transgenic lines were generated by injecting DNA for germline transformation into embryos of the w^{1118} strain of *D. melanogaster* using standard procedures (Rubin et al. 1982) and then stabilized by standard crossing scheme.

Protein extraction and western blots:

Frozen samples described above were homogenized in protease inhibiting homogenization buffer [PIHB; (Monsma et al. 1988)] with the addition of two phosphatase inhibitors, 20mM sodium fluoride and 10mM -glycerophosphate. Total protein was quantified by Bio-Rad Protein Assay (#500-0006, Bio-Rad Laboratories, CA). For phosphatase treatment, extracts were either treated with calf intestine alkaline phosphatase (CIAP) (Roche, 20U/μl) or incubated under the same conditions but without CIAP as controls as in(J Yu et al. 1999). Samples were prepared for gel electrophoresis by boiling in sample-buffer for 3 minutes, and 20-60 micrograms of protein were loaded into each lane of a 10.6% SDS polyacrylamide gel. Loading was adjusted as needed for the various antibodies used. Western blots were prepared as previously described (Monsma et al. 1988). Anti- (total) ERK, anti-phospho-ERK, anti-phospho-p38 or anti-phospho-JNK (Cell Signaling Technology, MA) were used at dilutions of 1:1000, 1:1000, 1:500, and 1:250, respectively, according to manufacturer's recommendations. I used anti- (total) ERK staining as a loading control when also probing for phospho-ERK [Chapter 2, (Sackton et al. 2007)]. Anti-α-tubulin antibody (Sigma, catalog #T5168) was diluted 1:10,000. Guinea-pig anti-GNU [gift of T. Orr-Weaver, (Lee et al. 2003)] was diluted 1:5000. Anti-YA (Liu et al. 1995) was diluted 1:1,000. Guinea-pig anti-SMG [gift of W. Tadros and H. Lipshitz (Tadros et al. 2007)] was diluted 1:10,000. After incubation with primary antibody overnight at 4°C, blots were probed with anti-rabbit, -guinea pig, or -mouse HRP secondary antibodies (Jackson Immunochemicals, PA) at a 1:2000 dilution, according to manufacturer's recommendation. Signals were detected with ECL or ECL-Plus

(GE Healthcare, United Kingdom).

Immunofluorescence:

MKP3 germline clone or Oregon R P2 females were mated to Oregon R P2 males for 0-15 minute collections of MKP3 germline clone or control embryos. Embryos fixed in methanol/heptane were rehydrated in PBST, and DROP1.1 (anti-sperm tail) antibody [gift of T. Karr, (Karr 1991)] or anti-α-tubulin antibody (Sigma, catalog #T5168) were used at 1:1000 or 1:100 dilutions, respectively, in PBST. Secondary was Alexa 488-conjugated anti-mouse (Invitrogen, Carlsbad, CA). DNA was stained with 10μg/ml propidium iodide. Fixed and stained samples were mounted in (75% glycerol containing 940mM *n*-propyl gallate) or washed in MeOH and mounted in 2:1 benzyl benzoate: benzyl alcohol (Theurkauf 1994). Eggs and embryos were analyzed using confocal microscopy (Leica TCS SP2 system equipped with an argon-krypton laser and coupled to a Leica DMRBE microscope). Leica software was used to collect images. Where appropriate, Leica software was used to project multiple optical sections into a single image and to overlay images.

Protein purification and mass spectrometry:

FLAG M2 immunoaffinity purification was modified from (Kraus et al. 1998), using buffers with two-fold higher concentrations of protease inhibitors and phosphatase inhibitors. Mature oocytes were isolated from roughly 750, 1200, or 1500 females by the blender method of (Page et al. 1997). Oocytes were spun down to pellet in 50ml conical tubes (2 min spins, setting 4, benchtop centrifuge). Laid 0-1hr embryos (roughly 9,000,12,000 or 24,000) were dechorionated in 50% bleach, washed in Egg Wash Buffer (Karr et al. 1986), frozen in liquid nitrogen, and stored at -80degC. Tissues were resuspended in 7ml Buffer 1 (Kraus et al. 1998), and dounce homogenized in a 15 ml vessel for 12+ strokes, then poured into a 15ml centrifuge tube and iced ~10min. Homogenate was spun for 20 min at 15KRPM, supernatant was

removed to a 15ml conical tube and an equal volume of Buffer 2 was added. 200ul anti-FLAG M2 resin slurry (Sigma, rinsed according to manufacturer's instructions) was added and the tube was rotated end over end at 4°C for 4-5 hrs. The resin was then washed four times with 10 ml each Wash buffer (4 min spins on #4 clinical centrifuge). Two elutions were performed in 1.5ml tubes (spins at 5000rpm 3-4min) with 150ul high salt Elution Buffer, 10 min on ice with flicking every 2 min. Sample buffer was added to resin to release remaining bound protein into solution for gel electrophoresis. Eluates and resin samples were run on 7.5% polyacrylamide gels alongside BSA protein standards, and stained with SimplyBlue SafeStain (Invitrogen). Bands with 0.5-1.0mg FLAG-YA protein were excised and submitted for trypsin digest and Nano-LC-MS-MS to the Cornell Proteomics Facility.

Fertility, fecundity, and hatchability assay:

Virgin females of each genotype described in the text and figure legends (3–5 days old, aged on yeasted food) were singly mated to wildtype males. Males were discarded after mating. Each day thereafter, for the next 5 or 9 days, females were transferred individually to new vials. The total number of eggs laid in each vial was counted daily (total eggs=fecundity). Fertility was determined by counting the total number of progeny from each vial over the 6 or 10-day period. Hatchability was calculated as the fraction of number of eggs laid / number of progeny eclosed per vial per day. Data was analyzed with a repeated measures design ANOVA using the statistical package R and the function LME.

3.3 Results and Discussion

cortex, sarah and *prage* are upstream of protein dephosphorylation upon egg activation:

Mated female flies mutant for any of the three female-sterile genes known to

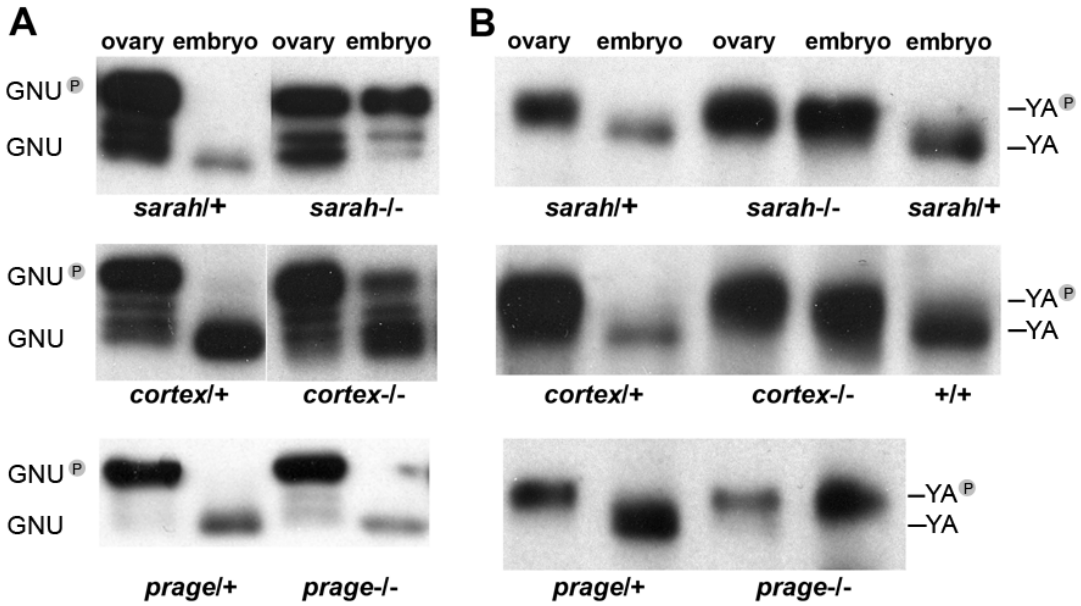
function in egg activation, *cortex*, *sarah*, and *prage,* lay fertilized embryos arrested at a gene-specific stage of meiosis (Chu et al. 2001; Horner et al. 2006; Tadros et al. 2003). These embryos also have defects in some other aspects of egg activation, such as translation of certain mRNAs. However, eggshell hardening and decreasing levels of phospho-MAPKs occur normally in these mutants. I examined the requirements for *cortex*, *sarah*, and *prage,* in YA and GNU dephosphorylation during egg activation by comparing the relative electrophoretic mobility of these two proteins in mature (stage 14) oocytes and early (activated) embryos on western blots. In embryos laid by *sarah* mutant mothers (hereafter "*sra* embryos"), the majority of the GNU protein has slower gel mobility, as in oocytes (Fig 3.1A), rather than faster gel mobility as in control embryos. Similarly, the YA protein in *sra* embryos has slower gel mobility than YA from control embryos, but slightly faster mobility than oocyte YA (Fig 3.1B). In embryos laid by *cortex* mutant mothers (hereafter "*cort* embryos"), a significant fraction of the GNU protein has slower gel mobility (oocyte-like) although the majority of the GNU protein in *cort* embryos has faster mobility (Fig 3.1A). The *sra* gel mobility phenotype is more pronounced for both GNU and YA than the *cort* gel mobility phenotype, in which YA and GNU have mobilities in embryos that are intermediate between their normal oocyte and embryo mobilities. The YA protein in *cort* embryos has slower gel mobility than YA from control embryos, but slightly faster mobility than oocyte YA (Fig 3.1B). As the developmental gel mobility differences in these two proteins are phosphatase-sensitive (Renault et al. 2003; Yu et al. 1999), the slower mobility forms of these two proteins are phosphorylated. There are intermediate mobilities for both YA and GNU, consistent with bioinformatic and genetic data (described above), and mass spectrometry data (described below) that multiple sites on these proteins can be phosphorylated. The phospho-GNU intermediates are visible as bands of GNU protein, since GNU is a small protein and

small changes in mobility due to phosphorylation can be distinguished. Because YA is a large protein the phospho-YA intermediates are more of a smear, and so bands of phospho-YA are best interpreted by noting both the position of the center of the band's intensity, and the upper and lower bounds of the band.

The YA/GNU dephosphorylation phenotype of embryos laid by *prage* mutant mothers (hereafter "*prg* embryos") is unlike that of *sra* and *cort* embryos, which both affect GNU and YA dephosphorylation, but to different extents. In *prg* embryos, the GNU protein is dephosphorylated as in control embryos (Fig 3.1A). However, YA is more phosphorylated in *prg* embryos than in control embryos, but less phosphorylated than in oocytes (Fig3.1B). Therefore, both *sarah* (calcipressin) and *cortex* (cdc20), but not *prage*, act upstream of GNU dephosphorylation upon egg activation. They could be regulating either a decrease in kinase activity, an increase in phosphatase activity, or both. The only kinases thus far proposed to phosphorylate GNU in vivo, the MAPKs (Zhang et al. 2004), decrease in activity upon activation independent of the egg activation genes *sra, cort* and *prg* (Sackton et al. 2007). Therefore, for *sra* or *cort* to be regulating a decrease in kinase activity upstream of GNU, another kinase would have to be the target of their regulation. The mechanisms and significance of GNU phosphorylation are not fully understood, so it is possible that an additional kinase could be involved, the activity of which would be inhibited upon egg activation downstream of *sra* and/or *cort*. PanGu phosphorylates GNU in vitro, but GNU's gel mobility is not affected in *png* mutant oocytes (Lee et al. 2003; Renault et al. 2003). As mentioned above, phosphatases could be upregulated upon egg activation, and GNU can be dephosphorylated by PP1 phosphatases in extracts, while PP2A has less of a role (Renault et al. 2003). It would be interesting to test whether any PP1 activities are altered in *sra* or *cort* mutant embryos. Whether *sra* and *cort* are upstream of the same or different GNU-regulating molecules is unclear, although the effect of

Figure 3.1 *sra* and *cort* function in GNU dephosphorylation, and *sra, cort* and *prg* function in YA dephosphorylation.

Western blot analysis was performed on total protein extracts of whole ovaries, 0-1-hr or 0-2-hr embryos from *sra*/ORP2, sra^{A108}/*sra*687, $cort^{QW55}$/Cy, $cort^{QW55}$/Df(2L)BSC9, ORP2, prg^{16A}/ FM6, or prg^{16A}/prg^{16A} females. Total protein loaded was not adjusted and therefore loading may be uneven from lane to lane. (A) Blots were probed with anti-GNU antibody. Ovarian GNU is phosphorylated and runs at a higher apparent molecular weight (~36kD) in both control and mutant samples. Embryo GNU is dephosphorylated in controls and runs at a lower apparent molecular weight (~33kD). In *sra* mutant embryos the GNU mobility is completely oocyte-like; GNU remains phosphorylated. In *cort* mutant embryos the GNU mobility is primarily embryo-like, but there is also some oocyte-like GNU consistently across experiments; a fraction of the GNU remains phosphorylated. In *prg* mutant embryos the GNU mobility is like control embryos; GNU is dephosphorylated. (B) Blots were probed with anti-YA antibody. Ovarian YA is phosphorylated and runs at a higher apparent molecular weight (~101kD) in both control and mutant samples. Embryo YA is dephosphorylated in controls and runs at a lower apparent molecular weight (~98kD). In *sra*, *cort*, and *prg* mutant embryos the YA mobility is intermediate between control oocyte and control embryo YA mobilities; YA is partially dephosphorylated. The highest apparent molecular weight (most phosphorylated) and lowest apparent molecular weight (least phosphorylated) forms of YA and GNU are marked on the sides of the blots, but intermediate degrees of phosphorylation are also visible on the blots. Western blots shown are representative of 3 or more independent replicates.


A
ovary embryo ovary embryo
GNU P
GNU
sarah/+ sarah-/-
GNU P
GNU
cortex/+ cortex-/-
GNU P
GNU
prage/+ prage-/-
B
ovary embryo ovary embryo embryo
–YA P
–YA
sarah/+ sarah-/- sarah/+
–YA P
–YA
cortex/+ cortex-/- +/+
–YA P
–YA
prage/+ prage-/-

lack of *sra* is significantly greater that lack of *cort*.

While YA is a MAPK target like GNU, it is also phosphorylated on multiple sites by other kinases (data shown below), which could be negatively regulated downstream of *sra*, *cort*, and *prg*. Both PP1 and PP2A phosphatases can dephosphorylate YA in extracts (Norene Buehner, unpublished results), so these and/or other phosphatases could be activated downstream of *sra*, *cort*, and *prg* upon egg activation.

No requirement for individual MAPKs for YA or GNU protein phosphorylation in oocytes:

Both the dephosphorylation of YA and GNU and the dephosphorylation/ inactivation of MAPKs occur at the oocyte-to-embryo developmental transition. To begin to test if this correlation was caused by a molecular relationship, I tested in oocytes whether inactivating the ERK pathway alone, or the JNK and p38 pathways combined, altered the phosphorylation levels of YA or GNU in oocytes, where MAPKs are active and YA and GNU are phosphorylated. This approach has the potential to identify which MAPK phosphorylates YA *in vivo*, and which phosphorylates GNU, if there is an *in vivo* specificity not reflected by *in vitro* studies. ERK (Yu et al. 2002), p38, and JNK (J. Cui, unpublished results) can all phosphorylate YA *in vitro*. Because null mutants of the MAPKs or their activating MAPKKs are lethal due to their many functions in various tissues, I used heat-shock induced FLP-FRT-mediated recombination to generate clones in the germline homozygous for mutant MAPKK(s). Such germline clones begin embryonic development, but have patterning defects and are embryo-lethal (Janody et al. 2000; Suzanne et al. 1999).

Germline clones lacking the MAPK kinase (MEK) upstream of ERK, *Dsor*, were collected as mature oocytes and analyzed by western blot. *Dsor* germline clone mature oocytes have YA with electrophoretic mobility comparable to control oocytes,

and the YA mobility can be increased (so it runs lower on the gel) by *in vitro* dephosphorylation with calf intestine alkaline phosphatase (CIAP) (Fig 3.2A). Therefore I also analyzed germline clones lacking the MAPK kinases upstream of JNK and p38, *hep* and *lic*, which are adjacent genes in Drosophila and are removed by a single small chromosome region deletion. *Hep/lic* germline clones were collected as mature oocytes and analyzed by CIAP treatment and western blot as described for *Dsor*. *Hep/lic* germline clone mature oocytes have phosphorylated YA with electrophoretic mobility comparable to that in control oocytes (Fig 3.2B).

Neither lack of ERK function alone, with active p38 and JNK present, nor lack of p38 and JNK function with active ERK present, prevents the dephosphorylation of YA. Two possible interpretations of this result are that MAPKs do not contribute to YA phosphorylation that causes slower electrophoretic mobility in oocytes, or that MAPK activities are redundant and so a YA gel mobility phenotype is obscured by the presence of remaining MAPKs when one or two are mutant. Counter to the first interpretation, mutation of a single MAPK site (T443A) causes a detectable difference in oocyte YA gel mobility (Yu et al. 1999), so phosphorylation of YA by MAPK probably does contribute to slower oocyte YA gel mobility. The difference in electrophoretic mobility between maximally-phosphorylated YA in wildtype oocytes, and YA that is not phosphorylated on its two MAPK sites (see below) while still being phosphorylated at nine other sites (see below) by other kinases, should be detectable as an intermediate mobility. However, if only a fraction of YA were migrating at this intermediate mobility because the MAPK(s) present compensate partially for the nonfunctional MAPK(s), the resulting smear would likely not appear to be consistently different between mutants and controls in multiple experiments. Similarly, if the active MAPK(s) compensate fully for the nonfunctional MAPK(s), there would be no mobility difference. Ideally, to address the issue of MAPK

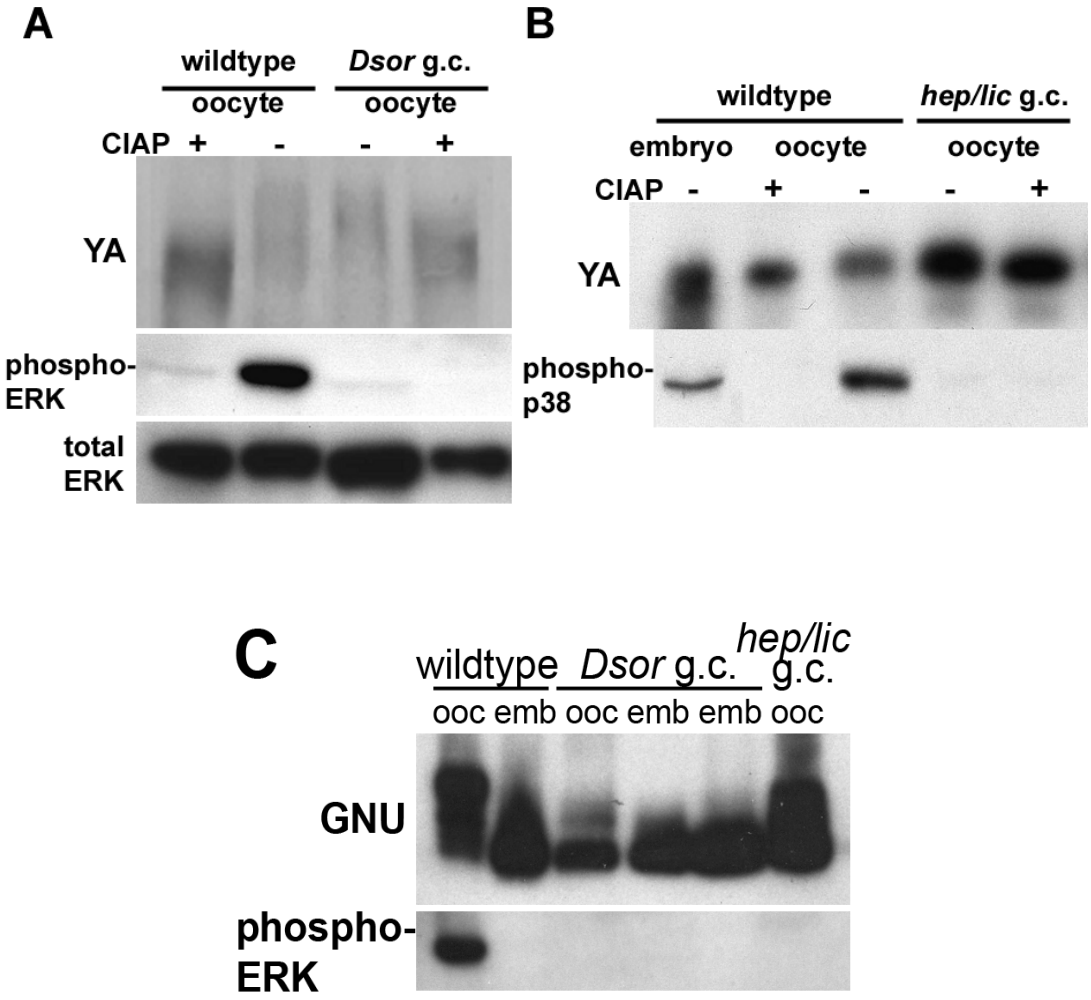
redundancy one would need to remove the function of all three MAPKs. Technically, it would be difficult or impossible to make germline clones simultaneously knocked out for *dsor, hep,* and *lic.* In addition, the use of RNAi is not feasible because it would be low efficiency with three interfering RNAs and incapable of knocking down the large quantities of maternal mRNA stored in the oocyte and early embryo. I explored the use of multiple pharmacological inhibitors or one pharmacological inhibitor in combination with germline clones, but because Drosophila egg activation occurs in the female reproductive tract as the egg is ovulated, drug delivery was an issue. *In vitro* activation would be amenable to pharmacological inhibitor use, but unfortunately the MAPK decrease is an aspect of egg activation that differs between *in vitro* and *in vivo* activation (Appendix C). If the ideal triple MAPK knockout had no effect on YA mobility, it would conflict with the altered electrophoretic mobility of MAPK site Ser/Thr to Ala mutants (Yu et al. 1999) and the detection by mass spectrometry of phosphorylation on MAPK sites (below). However, the redundancy of MAPKs explains the lack of change in YA mobility in *Dsor* and *hep*/*lic* germline clones, consistent with the aforementioned data supporting YA as a target of MAPKs in oocytes. Preliminary results show that *Dsor* germline clone mature oocytes have dephosphorylated GNU with electrophoretic mobility comparable to embryo GNU (Fig 3.2C), suggesting that phosphorylation by ERK is necessary for the normal phosphorylation state of GNU in oocytes, and the other MAPKs present do not phosphorylate GNU.

Mass spectrometry identification of YA phosphorylation sites:

In order to identify the different amino acid residues of YA that are phosphorylated *in vivo* in oocytes and embryos, I made transgenic flies expressing UASp-YA with a FLAG-tag (Sigma) on either the amino or carboxy terminus. Although YA with various other tags (HA, GFP) has previously been shown to be

Figure 3.2 MAPKK germline clone oocytes have wildtype YA phosphorylation levels.

Western blots of protein extracts from mature oocytes and 0-2-hr embryos from wildtype (ORP2) females or females whose germline tissues are clones homozygous for the mutant genotype indicated. These extracts were either treated with CIAP or incubated under the same conditions but without CIAP as controls. Anti-YA and Anti-GNU were used to detect YA and GNU proteins, respectively. YA and GNU reflect total protein levels loaded in each lane. (A) In germline clones of *Dsor,* the ERK-activating MAPKK, untreated oocyte YA has the same mobility as untreated wildtype oocyte YA (compare center two lanes). CIAP-treated oocyte YA from *Dsor* germline clones migrates faster on the gel, thus it has been dephosphorylated (compare two right lanes) like wildtype CIAP-treated oocyte YA (compare two right lanes). Anti-ERK was used to detect total ERK protein while anti-phospho-ERK was used to detect active ERK. The absence of phospho-ERK in the *Dsor* oocyte lanes is the expected *Dsor* mutant phenotype, confirming that the clones lack *Dsor* activity. (B) In germline clones lacking both *hep* and *lic,* the JNK- and p38- activating MAPKKs, respectively, untreated oocyte YA has the same mobility as untreated wildtype oocyte YA (compare third and fourth lanes). CIAP-treated oocyte YA from *hep/lic* germline clones migrates faster on the gel, thus it has been dephosphorylated (compare fourth and fifth lanes). Like wildtype CIAP-treated oocyte YA, it migrates similarly to embryo YA (compare lanes 1, 2, and 5). Anti-phospho-p38 was used to detect active p38. The absence of phospho-p38 in the *hep/lic* oocyte lanes is the expected *hep* mutant phenotype, confirming that the clones lack *hep* activity. (C) The slower mobility (phospho-) form of GNU is present in wildtype and *hep/lic* oocytes, but in *Dsor* oocytes and embryos most of the GNU is the same mobility as wildtype embryo GNU (dephosphorylated). Western blots shown are representative of 2 or more independent replicates.


A
wildtype
Dsor g.c.
oocyte
oocyte
CIAP + - - +
YA
phospho-ERK
total ERK
B
wildtype
hep/lic g.c.
embryo
oocyte
oocyte
CIAP - + - - +
YA
phospho-p38
C
wildtype
Dsor g.c.
hep/lic g.c.
ooc emb ooc emb emb ooc
GNU
phospho-ERK

nonfunctional (Liu et al. 1998), both my N-term (FLAG-YA) and C-term (YA-FLAG) FLAG-tagged YA constructs driven by *nanos- Gal4* could rescue the infertility of the Ya^2 mutant (which acts as a null). Males from at least four lines of UAS-FLAG-YA and UAS-YA-FLAG were crossed to X^XYa^2/Ya^+Y;;*nos*-Gal4/+ females to make female offspring with UAS-Gal4-driven expression of FLAG-YA or YA-FLAG without endogenous functional YA. All these female offspring were fertile, so FLAG-YA and YA-FLAG complement (rescue) the Ya^2 mutant infertility phenotype. To further test whether the transgenic FLAG-YA was behaving like endogenous YA, I treated FLAG-YA containing oocyte extracts with phosphatase (CIAP) at in Yu et al (1999) to determine if FLAG-YA was phosphorylated and also tested whether the electrophoretic mobility of FLAG-YA was developmentally regulated by comparing gel mobilities of oocyte and embryo FLAG-YA.

FLAG-YA from oocyte total protein extract treated with CIAP migrates faster that the same protein extract without enzyme treatment (Fig 3.3B left two lanes). Similarly, purified FLAG-YA treated with CIAP migrates faster on the gel than untreated controls (Fig 3.3 three lanes on right). Thus FLAG-YA behaves as a phosphoprotein. YA-FLAG from embryos migrates faster than YA-FLAG from embryos (Fig 3.3A), indicating that phosphorylation of YA-FLAG is developmentally regulated, as for endogenous YA. FLAG-YA phosphorylation is also developmentally regulated (data not shown). Much of the FLAG-YA was not eluted off the resin with FLAG peptide competitor and high salt, so I added denaturing sample buffer to the resin and boiled it. Loading the resulting supernatant onto the gel gave a strong band (Fig 3.3C) to analyze by tryptic digest and mass spectrometry.

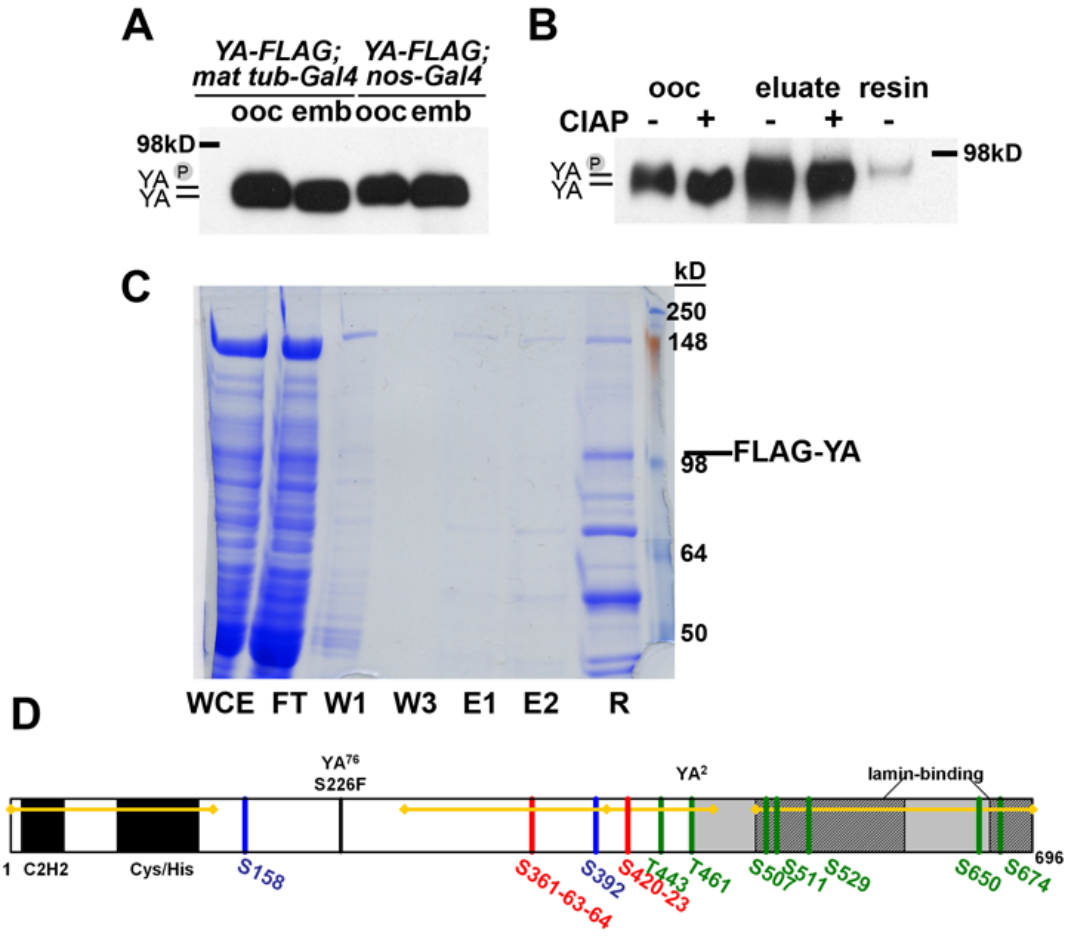
Three independent biological replicates each of FLAG-YA purified from oocytes and laid 0-1hr embryos were analyzed by the Cornell Proteomics Facility by nano-LC-MS-MS. Four of these six samples were additionally analyzed by

phosphoprotein enrichment on a Ti column before LC-MS-MS or by use of precursor ion scanning mode (instead of standard data acquisition) to detect only phosphopeptides. Based on the tryptic digest pattern of YA and the ionizability of the peptides produced, there was an average coverage of about 70% of the sequence of YA. At least eleven phosphorylation sites were detected on multiple peptides from more than one biological replicate, nine that are found in both oocyte and embryo samples, and two that are found only in oocytes, S158 and S392 (Figure 3.3D). For seven sites identified as phosphorylated in both oocytes and embryos; T443, T461, S507, S511, S529, S650 and S674, I am confident in the identification of the specific phosphorylated amino acid residue. Two other phosphopeptides found in both developmental stages each contain two or three potential phosphorylation sites, and it is not clear which of those sites in the phosphorylated one, or if more than one are phosphorylated. Specifically, S420, S423 or both are phosphorylated in both stages, and one or more of S361, S363, or S364.

The stoichiometry of phosphorylation at some sites can be estimated by comparing the area under the curve in the spectra of the phosphopeptide compared to the area under the curve in the spectra of the non-phosphorylated form of the same peptide (Britton et al. 2008; Tsay et al. 2000). Only some of the peptides are detected in both phosphorylated and non-phosphorylated forms, and both forms are necessary for comparison. For example, those phosphopeptides detected by Ti enrichment for phosphopeptides or precursor ion scanning are only detected in phosphorylated form because of the method used, and relative phosphorylation cannot be estimated. Of course, only the phospho form of some peptides are detected by regular LC/MS/MS as well. By this measure of the oocyte-specific phosphorylations, S158 has a relatively high frequency of phosphorylation, measured in two independent instances as 26.5% and 34.7%. In contrast, S392 was only measurable once, and its frequency was 2.4%.

Figure 3.3 Phosphorylation sites of YA in oocytes and embryos.

Western blot analysis was performed on total protein extracts or purified protein from mature (stage 14) ovarian oocytes and 0-2-hr embryos from females expressing transgenic YA-FLAG (A) or FLAG-YA (B, C) driven by one or both of the germline drivers indicated. Anti-YA and was used to detect YA protein. (A) FLAG-tagged YA protein has slower electrophoretic mobility in oocytes than embryos, as is typical of endogenous YA. (B) Oocyte extracts or purified protein were either treated with calf intestine alkaline phosphatase (CIAP) or incubated under the same conditions but without CIAP as controls. Total oocyte extract FLAG-YA is dephosphorylated by CIAP, as is purified FLAG-YA eluted off the column. FLAG-YA stuck to the resin has the slower mobility of phosphorylated YA. (C) Colloidal coomassie-stained gel of a representative FLAG-YA purification. WCE= whole cell extract, FT=flowthrough, W1 and W3 are washes, E1 and E2 are elutions, and R is the resin after elutions. (D) Diagram of the positions of YA's phosphorylated residues in relation to predicted functional domains and mutant forms of YA. Grey at the C-term indicates the region deleted in the Ya^{2} truncation, yellow lines indicate chromatin-binding regions. The two darker grey regions near the C term are lamin-binding regions, and the two black regions near the N term are cysteine/histidine regions. The site of the Ya^{76} point mutation is also indicated. Oocyte-specific phosphorylation sites are labelled in blue, those found in both oocytes and embryos are green if a single residue has been identified and red if the precise residue phosphorylated is unknown.


A
YA-FLAG; mat tub-Gal4
YA-FLAG; nos-Gal4
ooc emb ooc emb
98kD
YA P
YA
B
ooc
eluate
resin
CIAP - + - + -
98kD
YA P
YA
C
kD
250
148
98
64
50
FLAG-YA
WCE FT W1 W3 E1 E2 R
D
YA76 S226F
YA2
lamin-binding
1 C2H2
Cys/His
S158
S361-63-64
S392
S420-23
T443
T461
S507
S511
S529
S650
S674
696

Quantifications of other YA phosphorylation sites are included in Table 3.1. Nine additional phosphopeptides or phosphosites were detected by the mass-spectrometry analysis that occurred only once (or only once per developmental stage) and so they cannot be confidently defined as oocyte-specific, embryo-specific, or found in both. Assuming the relative level of phosphorylation is reflected by the frequency of detection, if the sites that have not been confirmed by multiple biological replicate actually occur in vivo, they may have less biological significance than more highly phosphorylated sites. By submitting the protein sequence of YA to the scansite motif-scanning program (www.scansite.mit.edu) (Obenauer et al. 2003), kinases that could phosphorylate some of the residues I detected have been suggested by the presence of their consensus target motifs in the amino acid sequences surrounding these particular phosphosites. The kinases GSK3, cdc2, cdk5, polo, CAMKII and PKCmu were suggested by this analysis as candidates for further study. A role for PanGu kinase, which might be a candidate YA kinase because it is present in oocytes, was not tested by the bioinformatic search for kinase consensus target motifs because no such motif is known for PanGu. The majority of the phosphosites are highly conserved across the thirteen Drosophila species for which *Ya* gene sequences are available (Appendix), and these sites are present in short stretches of sequence that are moderately to highly conserved (Table 3.1).

I found more sites phosphorylated in oocytes than embryos, consistent with the slower electrophoretic mobility of oocyte YA than embryo YA. Future studies of the relationship between YA's subcellular localization and its phosphorylation will focus on these sites. S158 is predicted to be a GSK3 target, but I did not detect phosphorylation of T162, which is the predicted priming phosphorylation for GSK3 targeting of S158. Other kinases are predicted to phosphorylate sites that are phosphorylated in both oocytes and embryos. Both CaMKII and PKCμ are predicted

to be able to phosphorylate S507, although whether either of these actually phosphorylates YA in vivo remains to be shown. CaMKII has a role in regulating the resumption of meiosis during egg activation in Xenopus and mice (Knott et al. 2006), so it may act during egg activation in Drosophila downstream of the initial calcium signal for activation. Polo kinase is predicted to phosphorylate S650 (Elia et al. 2003; Nakajima et al. 2003), which is the phosphorylated residue that is least conserved between Drosophila species, since it is found in only three out of thirteen species, and the residues immediately surrounding it are also more diverged than conserved. Polo kinase has been shown to interact with YA in yeast two hybrid assays (J. Cui, unpublished results), and polo is required for aspects of oocyte meiosis (Riparbelli et al. 2000). Of the five MAPK sites in YA, T443 and S674 are phosphorylated in both oocytes and embryos, T443 with low frequency (0.1% to 6% in four quantifications) and S674 with the second highest frequency of any site quantified (68% and 76% in two quantifications). As mentioned previously, the T443A mutation changes the gel mobility of oocyte YA (J Yu et al. 1999), demonstrating that it is a phosphorylated amino acid residue, although a difference between T443A and control embryo mobility was too small to detect, though it would be predicted by the mass spectrometry data. Multiple non-phosphorylated peptides from both developmental stages were detected for T162 and T489, so they are quite likely not phosphorylated, but T263 is in a region of poor coverage, so only one non-phosphorylated peptide from each developmental stage was detected, suggesting only inconclusively that it is not phosphorylated.

Germline clones lacking the phosphatase MKP3 do not have egg activation defects:

The activity of MAPKs is regulated by phosphorylation of a tyrosine residue on a flexible regulatory region of the protein that undergoes a conformational shift to

Table 3.1 YA phosphorylation sites in oocytes and embryos

Site: the amino acid reisdue(s) identified as phosphorylated by mass spectrometry. Found in: which biological samples contained the identified phospho-site. Quantification (% phosphorylation): signal intensity (area under the curve) for the phosphopeptide in which the phospho-site was identified was compared to signal intensity (area under the curve) for the non-phosphorylated form of the same peptide. When only phosphopeptides were detected, this estimated quantification was not determinable (n.d.), suggesting that residue was phosphorylated in the majority of the protein present (~100%). Conserved: of the thirteen Drosophila species whose YA protein sequences are aligned in Appendix A, the number in which the Ser/Thr residue is identical is indicated. If adjacent amino acids are also conserved, the conserved amino acid sequence is included. Predicted kinase: Bioinformatic predictions of kinase target consensus motifs suggest the kinases in this column as likely to phosphorylate the residues identified as phosphorylated.

Site	Found in	Quantification (% phosphorylation)	Conserved	Predicted kinase
S158	oocyte	26.5%, 34.7%	13/13 **S**TQTSP	GSK3
S361, S363, S364	oocyte & embryo	~30% average in both	13/13, 9/13, 10/13	
S392	oocyte	2.4%	13/13 **S**AQK	
S420/423	oocyte & embryo	n.d.	13/13 NSCESVEII	
T443	oocyte & embryo	oocyte 6%, 5.7% embryo 0.1%, 3.6%	13/13 PxxTPI	MAPK
T461	oocyte & embryo	n.d.	13/13 IKFE**T**P	cdc2
S507	oocyte & embryo	n.d.	13/13 IVPRQL**S**G	CaMKII, PKCμ
S511	oocyte & embryo	n.d.	10/13 **S**P	cdk5
S529	oocyte & embryo	oocyte n.d. embryo 82%	8/13	
S650	oocyte & embryo	oocyte 4%, 19% embryo 7%, 7%, 14%, 18%	3/13	polo
S674	oocyte & embryo	oocyte 68% embryo 75.7%	13/13 **S**P	MAPK

obstruct the kinase active site when the protein is not phosphorylated (Canagarajah et al. 1997). One type of Tyr phosphatase that can catalyze this dephosphorylation are the MAP kinase phosphatases, or MKPs. In Drosophila, germline clones lacking the phosphatase MKP3 have been shown to arrest as very early embryos with one or two nuclei (Kim et al. 2004). This is striking phenotype for mutating one of eight predicted MKPs in the Drosophila genome (Morrison et al. 2000), although the developmental and tissue expression patterns of all eight have not yet been determined; there may be fewer than eight expressed in the oocyte. Using the lines from Kim et al (2004), I made germline clones and confirmed the phenotypes of shorter egg length and embryogenesis arrest they described in that paper.

I tested whether this embryogenesis arrest involved misregulation of MAPK levels, and if any aspects of egg activation are disrupted in MKP3 germline clones. All egg activation events I assayed occurred normally in MKP3^{-} germline clones. GNU is dephosphorylated in MKP3^{-} germline clone embryos (Fig 3.4B). Smaug is translated only upon egg activation in MKP3^{-} germline clones (Fig 3.4C) as in wildtype embryos. Vitelline membrane protein crosslinking also appears to occur normally because I saw no premature embryo lysis in bleach during sample preparation for western blots and immunofluorescence. Interestingly, ERK is dephosphorylated in MKP3^{-} germline clone embryos upon egg activation (Fig 3.4A), as is p38 (data not shown). The dephosphorylation of ERK in the absence of MKP3 function suggests that MKP3 is not necessary for ERK dephosphorylation upon egg activation, probably due to redundant MKP or other dual-specificity phosphatase activity.

In order to further characterize the arrest point of MKP3^{-} germline clones, I stained them with antibody against the sperm tail to verify that they can be fertilized and with anti- tubulin to visualize spindles and other microtubule structures. MKP3^{-} germline clones eggs can be fertilized (Fig 3.5A). Meiosis resumption appears normal,

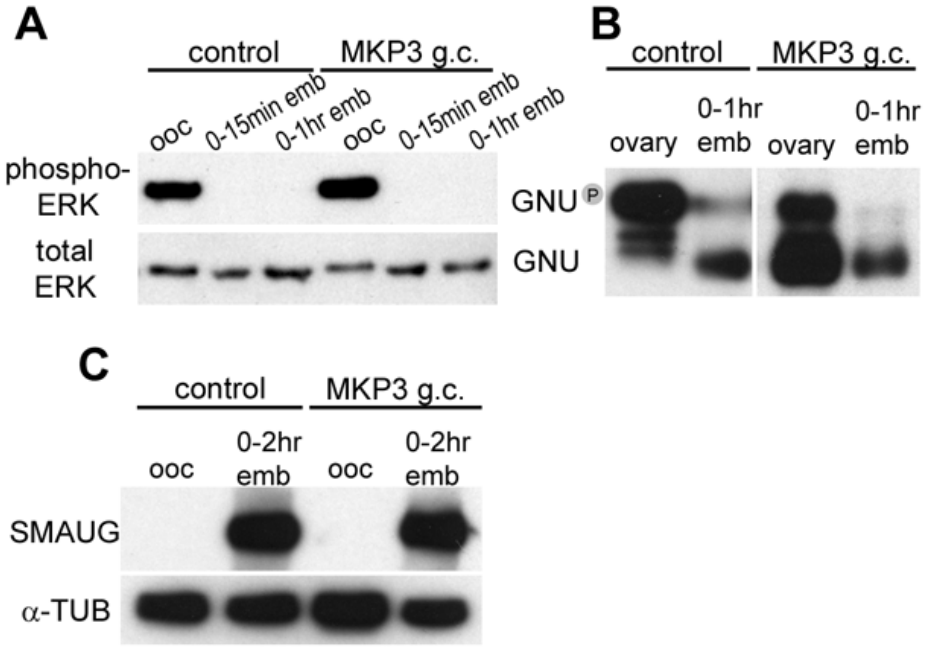


Figure 3.4 Three aspects of egg activation are normal in MKP3 germline clones. Western blots performed on total protein extracts of mature oocytes, whole ovaries, and embryos of the indicated collection lengths from wildtype (ORP2 or fertile sibling controls) females or females whose germline tissues are clones homozygous for MKP3. (A) Dephosphorylation of the MAP kinase ERK upon egg activation was assayed by western blotting with anti-phospho-ERK antibody. Anti-total ERK was used as a loading control. Phospho-ERK levels decrease upon egg activation in MKP3 germline clone embryos as in controls. (B) Dephosphorylation of GNU upon egg activation was assayed by western blotting with anti-GNU antibody. Phosphorylated GNU in MKP3 germline clone oocytes has a slower electrophoretic mobility than dephosphorylated GNU in MKP3 germline clone embryos, as in wildtype egg activation. (C) Translation of the SMAUG protein after egg activation was assayed by western blotting with anti-SMG antibody. Anti-α-tubulin antibody was used as a loading control. SMG levels in 0-2hr-old embryos increase normally in MKP3 germline clones.

although in MKP3^{-} germline clone embryos, the meiotic spindles are not always aligned end to end as is typical of meiosis II in Drosophila, and their DNA is not positioned properly on the metaphase plate or in anaphase (Fig 3.5C). However, the presence of embryos with five nuclei (four female-derived and the sperm nucleus) suggests that meiosis can sometimes complete in the absence of MKP3 (n=4) (Fig 3.5B). When fewer than five nuclei are seen in fixed embryos they may be actively meiotic, or products of incomplete meiosis, or the result of nuclear associations after meiosis as seen in *Ya* mutant embryos (Chapter 5). The *MKP3* mutant phenotype more closely resembles the post-activation arrest of *Ya* mutants (Chapter 5) than the egg activation arrest of mutants such as *sarah or cortex*, which do not complete meiosis and have defects in other aspects of egg activation.

Constitutively active ERK does not disrupt egg activation:

Although attempts to inactivate a particular phosphatase or class of phosphatases could prove fruitful in detecting a significant function that a phosphatase serves in egg activation, often issues of phosphatase activity redundancy will obscure such analyses. Trying to observe the effect of blocking one particular protein dephosphorylation that occurs upon activation, the dephosphorylation of ERK, the phosphatase I inactivated by using a germline clone mutant was MKP3. Since ERK was still dephosphorylated, and thus inactivated in MKP3^{-} embryos, I sought a different way to test the role of ERK dephosphorylation (if any) in egg activation events. To learn the consequences of the presence of active ERK in embryos, I ectopically expressed a constitutively active form of ERK in embryos and tested for its role in activaton. The “sevenmaker” allele of ERK (rl^{Sem}) is a single amino acid substitution (D334N) that creates a constitutively active ERK (Brunner et al. 1994). I made transgenic flies expressing rl^{Sem} with two different sets of 5' and 3' regulatory regions. First, in order to use the UAS-GAL4 controlled expression system, I made

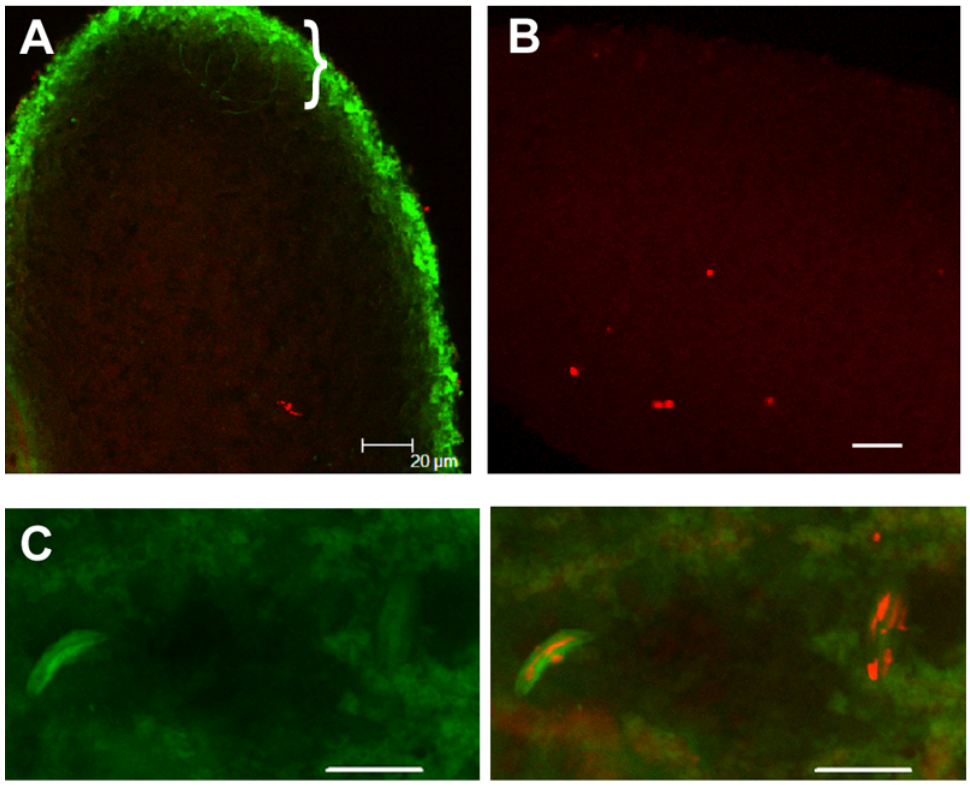


Figure 3.5 The arrest point of MKP3 germline clones is after fertilization and before the first mitosis.

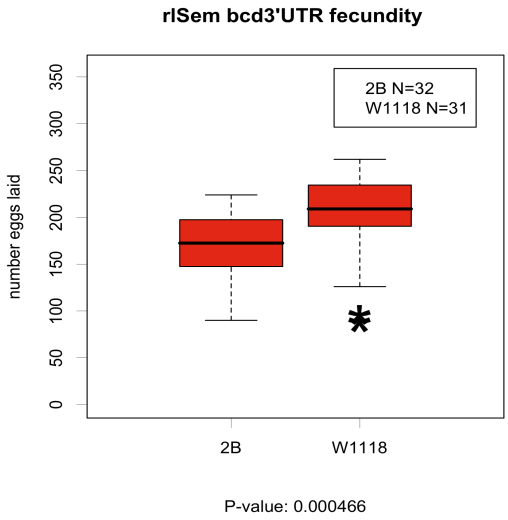
Immunostaining of 0-1 hour old MKP3 germline clone embryos. DNA shown in red. (A) Anti-sperm tail antibody shown in green. The presence of the sperm tail coiled inside the embryo (}) indicates fertilization has occurred. (B) Five nuclei is the maximum number of nuclei seen in MKP3 germline clone embryos. The four nuclei at the bottom of the panel could be the female meiotic products in telophase II or later, in which case the uppermost nucleus would be the male pronucleus. (C) Anti-tubulin staining shown in green. These meiotic spindle figures in MKP3 germline clone embryos have chromosomes dispersed along the spindle (left spindle) and off the spindle (right spindle). A-C are projections of multiple confocal images. Bar=20µm in A,B, Bar=10µm in C.

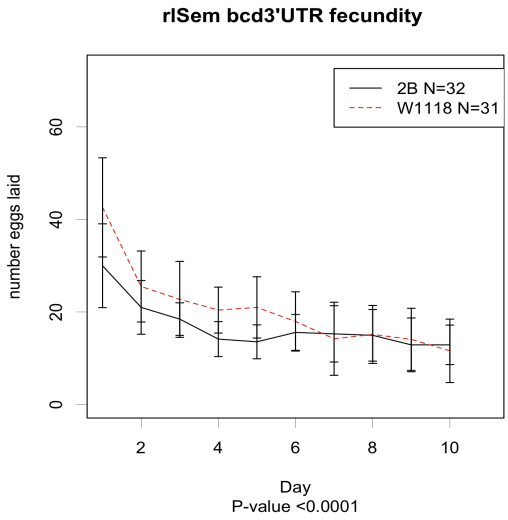
UASp-rl^{Sem} (see Materials and Methods). The UASp promoter works better for expression in the germline than the more common UASt (Rørth 1998). The GAL4 drivers we have available to express proteins during egg activation, *nanos-GAL4* and *maternal tubulin-GAL4*, are expressed in oocytes and their mRNAs and proteins persist and/or are translated in embryos. Second, in order to restrict expression of the constitutively active ERK to embryos and egg activation, to avoid confounding effects in oocytes that might subsequently affect egg activation, I made transgenic flies with the promoter and 5'UTR of the *gnu* gene and the 3'UTR of the *bcd* gene. A construct using the promoter and 5'UTR and the coding sequence of *gnu* with the 3'UTR has been used to express GNU with the temporal and spatial patterning of Bicoid (Zhang et al. 2004). I substituted the coding sequence of rl^{Sem} into the same construct (details in Materials and Methods). The *gnu* promoter and 5'UTR will promote transcription during oogenesis, when *gnu, rl,* and *bicoid* are all normally transcribed. The *bcd* 3'UTR (Gottlieb 1992) will restrict translation of the maternally-provided construct mRNA until after egg activation.

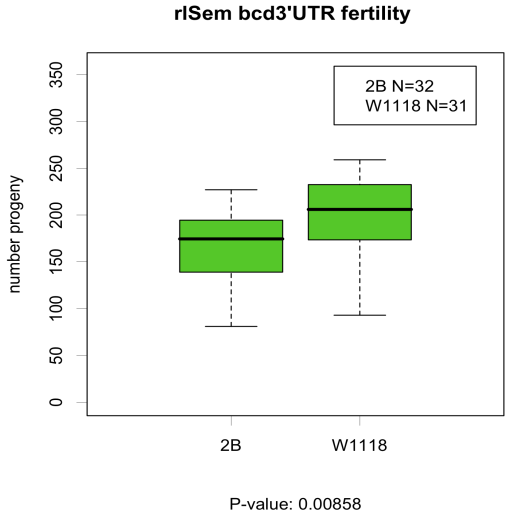
When this constitutively active ERK is present either under the control of the bcd 3'UTR in embryos (Fig 3.6) or driven by the UAS-Gal4 system in the germline and persisting in embryos (Fig 3.7), the females have subtle but statistically significantly lower numbers of egg laid than genetically matched control females (w^{1118} and w^{1118};Gal4 respectively). From rl^{Sem} bcd 3'UTR females, the number of progeny is also subtly though statistically significantly lower, and thus there is no difference in hatchability (Fig 3.6). However, for embryos from females where UAS-rl^{Sem} is driven by *nanos*-Gal4 hatchability is significantly lower (28%, 69%) than controls (96%). In females where UAS-rl^{Sem} is driven by *maternal tubulin-Gal4*, hatchability is very low to zero. The stronger hatchability phenotype seen with the *maternal-tubulin-Gal4* driver might be expected if it causes higher expression levels.

Figure 3.6 A normal fraction of eggs hatch when transgenic *rl*^{Sem} is expressed in embryos under the regulation of the *bcd* 3'UTR.

The numbers of eggs laid per day and the number of those eggs that developed into adult flies were counted for *rl*^{Sem}*bcd3'UTR* females (2B in legends) and *w*^{1118} control females (W1118 in legends). There is a slight but statistically significant difference between the number of eggs laid by the transgenic and the control females, but a similar fraction of eggs hatch for both lines, so there is also a slight but statistically significant difference between the number of progeny from transgenic and control females. I did one experimental replicate. The p-values shown are significant for comparisons between control and transgenic for fecundity and fertility, but not hatchability.


rlSem bcd3'UTR fecundity
2B N=32
W1118 N=31
number eggs laid
0
50
100
150
200
250
300
350
2B
W1118
P-value: 0.000466


rlSem bcd3'UTR fecundity
2B N=32
W1118 N=31
number eggs laid
0
20
40
60
2
4
6
8
10
Day
P-value <0.0001


rlSem bcd3'UTR fertility
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W1118 N=31
number progeny
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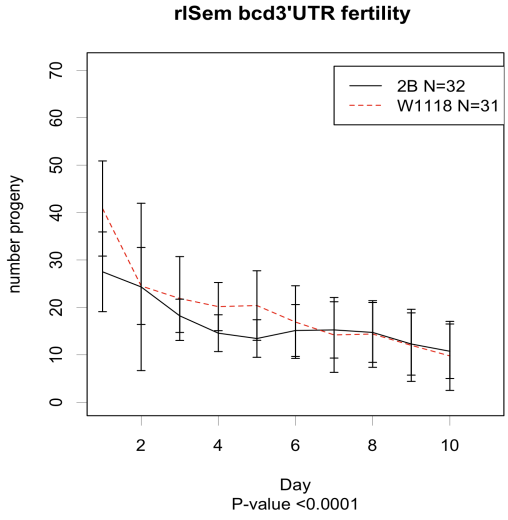
50
100
150
200
250
300
350
2B
W1118
P-value: 0.00858


rlSem bcd3'UTR fertility
2B N=32
W1118 N=31
number progeny
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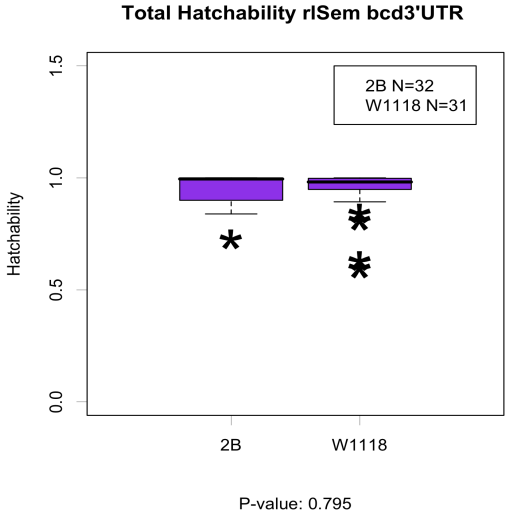
10
20
30
40
50
60
70
2
4
6
8
10
Day
P-value <0.0001


Total Hatchability rlSem bcd3'UTR
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W1118 N=31
Hatchability
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0.5
1.0
1.5
2B
W1118
P-value: 0.795

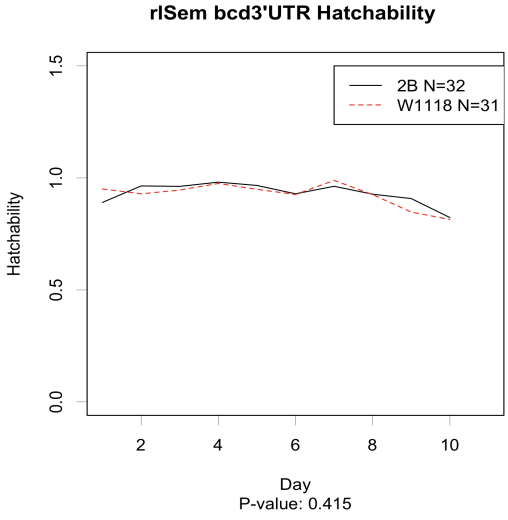
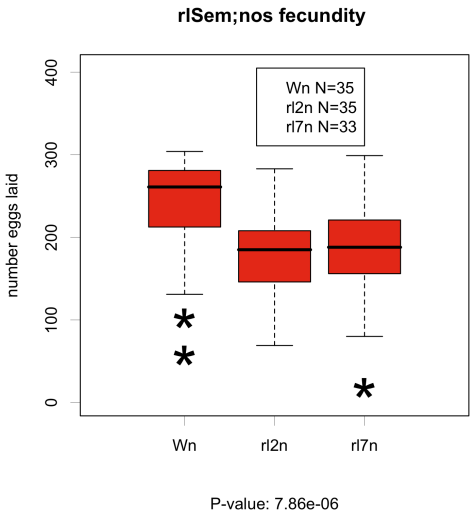

rlSem bcd3'UTR Hatchability
2B N=32
W1118 N=31
Hatchability
0.0
0.5
1.0
1.5
2
4
6
8
10
Day
P-value: 0.415

Figure 3.7 Fewer eggs hatch when transgenic *rl*^{Sem} is expressed in oocytes and embryos under UAS-GAL4 regulation.

The numbers of eggs laid per day and the number of those eggs that developed into adult flies were counted for *UASp-rl*^{Sem};*nos-Gal4* females (2 lines, rl2n and rl7n in legends) and *w*^{1118};*nos-Gal4* control females (Wn in legends). There is a statistically significant difference between the number of eggs laid by the transgenic and the control females, but an even greater difference between the number of progeny, so there is a statistically significant difference in hatchability. I did one experimental replicate. The p-values are significant at <0.0001 for all comparisons between controls and transgenics for fecundity, fertility, and hatchability (except for Wn vs. rl7 fecundity, which was significant at 0.0003).



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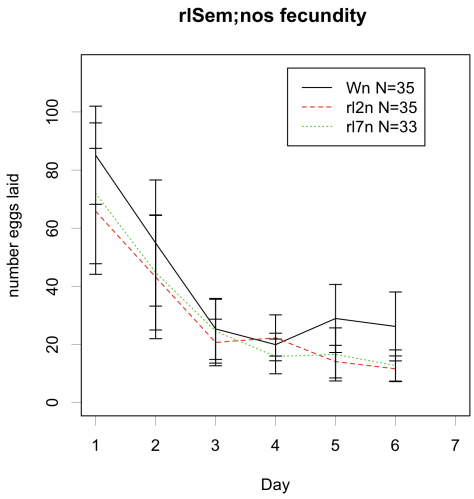
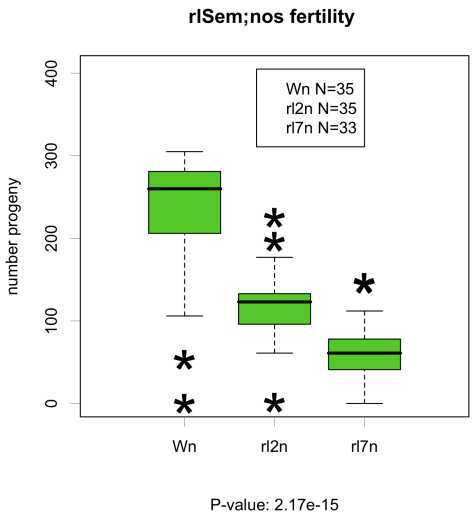
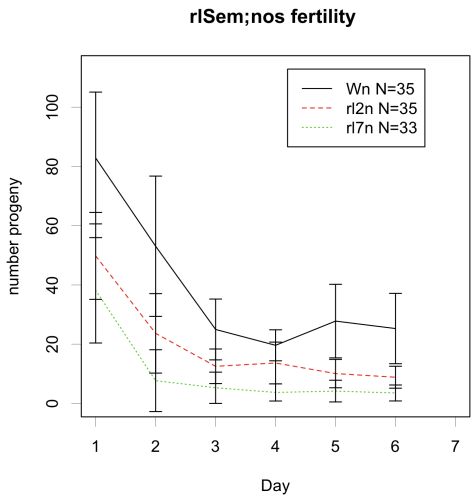
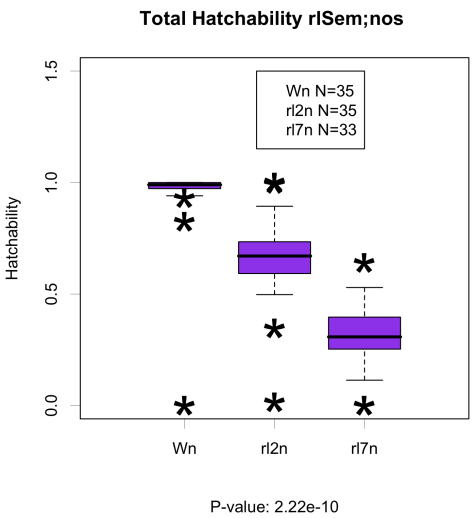


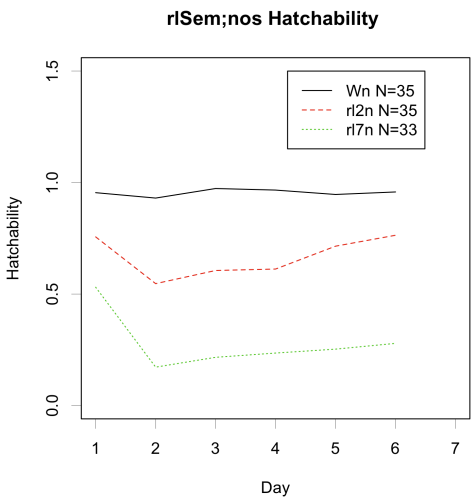


P-value: 2.17e-15





P-value: 2.22e-10



I will be testing for the presence of the rl^{Sem} transcript by RT-PCR and sequencing, since the protein is untagged and therefore cannot be distinguished from endogenous protein by western blot but this method cannot detect relative levels of the endogenous and *Sevenmaker* alleles, except that there must be significant levels of both sequences for the single nucleotide "polymorphism" to be detected. When this constitutively active ERK is present either in embryos only under the control of the bcd 3'UTR or driven by the UAS-Gal4 system in the germline and persisting in embryos, the dephosphorylation of GNU and SMG translation both occur normally in embryos (Fig 3.8). There is no obvious effect of UASp-rl^{Sem} on specific events of egg activation. This construct is only increasing ERK activity, so p38 and JNK are inactivated normally by dephosphorylation upon egg activation in these lines. Therefore p38 or JNK targets, or targets sensitive to total MAPK activity levels, would not be affected by rl^{Sem}.

Inhibiting PP1 phosphatases causes egg activation defects:

There are four Drosophila genes for catalytic domain proteins of the PP1 class of Ser/Thr phosphatase (Kirchner et al. 2007). Because of the redundancy, PP1s are not amenable to traditional single-mutant studies. There are two proteins known to inhibit PP1 activity, Nipp (nuclear inhibitor of PP1) and Inhibitor 2 (I2Dm), which when over-expressed under the UAS-Gal4 system will inhibit PP1 phosphatases (Bennett et al. 2003). Their normal role is to prevent inappropriate dephosphorylation events, but when over-expressed they more broadly inhibit PP1 activity. To test for the role of PP1s in egg activation, I drove over-expression of these two inhibitors by UAS-Gal4 in the germline. Previous studies of PP1 inhibitor over-expression used UASt-Nipp and UASt-I2Dm in somatic tissues (Bennett et al. 2003), but UASt does not function well in the germline. I made transgenic flies with each of these inhibitors under the UASp promoter for robust germline expression (Rørth 1998). I crossed

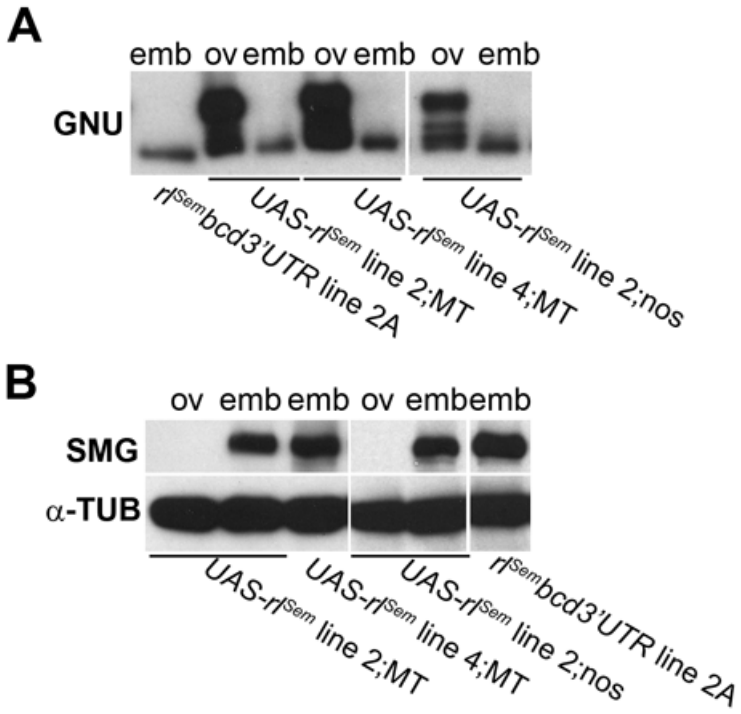


Figure 3.8 Two aspects of egg activation are normal when transgenic *rl^{Sem}* is expressed.

Western blot analysis was performed on total protein extracts of whole ovaries and 0-1-hr (for GNU) or 0-2-hr (for SMG) embryos from transgenic *UAS-rl^{Sem};mt-Gal4, UAS-rl^{Sem};nos-Gal4, or rl^{Sem}bcd3'UTR* females. The same *UAS-rl^{Sem}* transgenic line was used crossed to either the *maternal tubulin-* or *nanos- Gal4 driver lines.* (A) Dephosphorylation of GNU upon egg activation was assayed by western blotting with anti-GNU antibody. Phosphorylated GNU in transgenic oocytes has a slower electrophoretic mobility than dephosphorylated GNU in transgenic embryos, as in wildtype egg activation. (B) Translation of the SMAUG protein after egg activation was assayed by western blotting with anti-SMG antibody. Anti-α-tubulin antibody was used as a loading control. SMG levels in 0-2hr-old embryos increase normally in transgenics.

homozygous transgenic UAS-inhibitor flies with flies homozygous for either of two female germline drivers, *nanos-Gal4* or *maternal tubulin-Gal4*.

If inhibition of PP1s affects egg activation, a decrease in hatchability is expected in eggs with the inhibitors (fewer larvae hatch from eggs). Infertile egg activation mutants lay normal numbers of eggs with 0% hatchability, and a hatchability defect is a less severe but related phenotype. If PP1 inhibitors affect oogenesis, a decrease in fecundity is expected (fewer eggs are laid), and the laid eggs may have visible defects. I performed large-scale (at least 30 females per line) quantitative experiments using the *nanos-Gal* driver, and also made qualitative observations of small-scale (six or fewer females per independent transgenic line) experiments using the *maternal tubulin-Gal* driver. The small-scale qualitative experiments are not conclusive alone, but are consistent with the large-scale experiments, and so they are described here to supplement and support the large-scale experiments. Oocytes and laid embryos of *UASp-Nipp; maternal tubulin-Gal4* (*Nipp;MT*) females seem unhealthy; a small fraction of oocytes seem stunted or otherwise abnormal, and a significant fraction of laid embryos are short and/or translucent the day they are laid, (instead of opaque white). The oocytes and embryos of *UASp-I2Dm; maternal tubulin-Gal4* (*I2Dm;MT*) females have no obvious morphological defects the day they are laid. Females with either *UASp-I2Dm* or *UASp-Nipp* driven by *maternal tubulin-Gal4* have very few progeny compared to wildtype females. Totaling progeny hatched in ten days by two lines each, *I2Dm;MT* females had an average of 0-5 progeny/female, and *Nipp;MT* females had an average of 6-26 progeny/female, while wildtype females would typically have hundreds of progeny in ten days. For the *Nipp;MT* females the reduced fertility seems to be due to a combination of reduced fecundity and reduced hatchability based on my small-scale semi-quantitative observations. These females laid only an average of 16-42

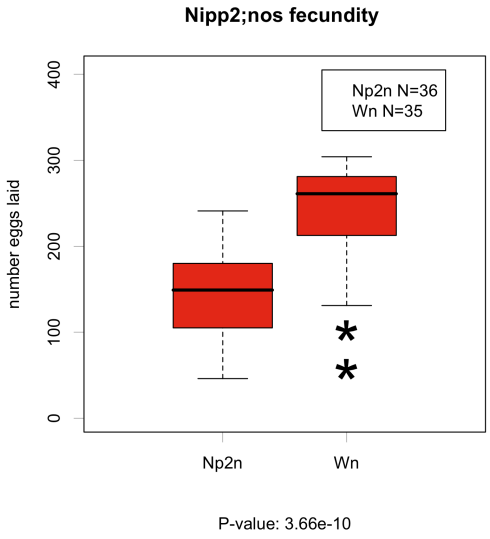
eggs/female in the first two days (wildtype would lay ~50-200), which is reduced fecundity, but the number of progeny hatched is sill low relative to embryos laid. For the *UASp-I2Dm; maternal tubulin-Gal4* females the reduced fertility seems to be due primarily to significantly reduced hatchability based again on small-scale semi-quantitative observations. These females laid an average of 49-57 eggs/female in the first two days, which is the low end of normal fecundity, but they had almost no progeny, thus very low hatchability. After three days observation of embryos in any given vial showed that some of the embryos that had not hatched had turned brown inside, with a translucent chorion. I interpret the brown color as a characteristic of dead or dying fertilized embryos, and the white eggs that are three days old as unfertilized or fertilized with very early embryonic arrest.

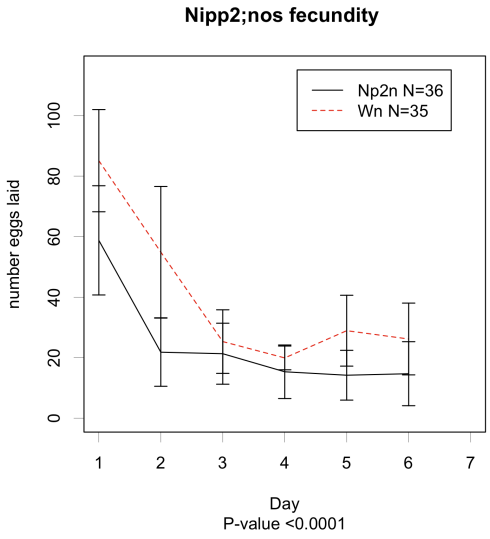
Based on my data on *UASp-FLAG-Ya* transgenic lines, *maternal tubulin-Gal4* causes reduced hatchability of FLAG-YA-expressing embryos while *nanos-Gal4* does not. In these lines I compared expression levels of protein between *nanos-Gal4* and *maternal tubulin-Gal4* drivers by anti-FLAG western blot, and the *maternal tubulin-Gal4*-driven expression is consistently higher than *nanos-Gal4* -driven expression (data not shown). Therefore I examined the effect of driving *UASp-I2Dm* or *UASp-Nipp* with *nanos-GAL4*, in order to vary the amount of PP1 inhibitors, since less inhibitor expression might reduce any oogenesis defects that lead to low fecundity.

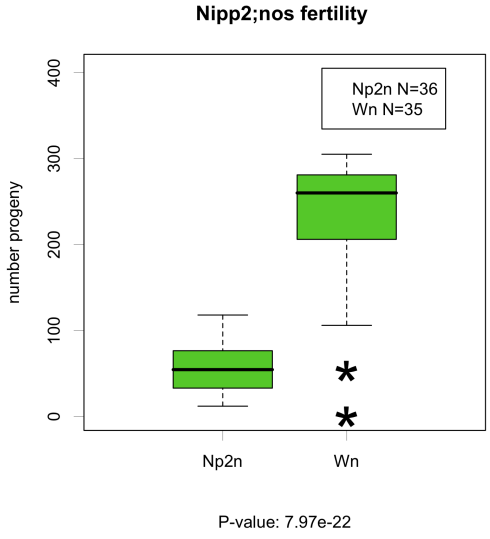
I assayed the fertility (number of progeny), fecundity (number of eggs laid), and hatchability (fertility/fecundity) of *UASp-Nipp* driven by *nanos-Gal4*. These *UASp-Nipp*;*nanos-Gal4* females have statistically significantly lower fecundity and fertility than w^{1118}; *nanos-Gal4* genetically-matched control females, and significantly reduced hatchability (Fig 3.9). The fecundity defect, which reflects an oogenesis defect, was less severe than for *Nipp;MT* females, since *UASp-Nipp*;*nanos-Gal4* females laid an average of 81 embryos/female in the first two days, more than

Figure 3.9 Fewer eggs hatch when transgenic *Nipp* is expressed in oocytes and embryos under UAS-GAL4 regulation.

The numbers of eggs laid per day and the number of those eggs that developed into adult flies were counted for *UAS-Nipp*;*nos-Gal4* females (Np2n in legends) and w^{1118};*nos-Gal4* control females (Wn in legends). There is a statistically significant difference between the number of eggs laid by the transgenic and the control females, but an even greater difference between the number of progeny, so there is a statistically significant difference in hatchability. The p-values are significant at <0.0001 for all comparisons between controls and transgenics for fecundity, fertility, and hatchability.


Nipp2;nos fecundity
Np2n N=36
Wn N=35
number eggs laid
0
100
200
300
400
Np2n
Wn
P-value: 3.66e-10


Nipp2;nos fecundity
Np2n N=36
Wn N=35
number eggs laid
0
20
40
60
80
100
1
2
3
4
5
6
7
Day
P-value <0.0001


Nipp2;nos fertility
Np2n N=36
Wn N=35
number progeny
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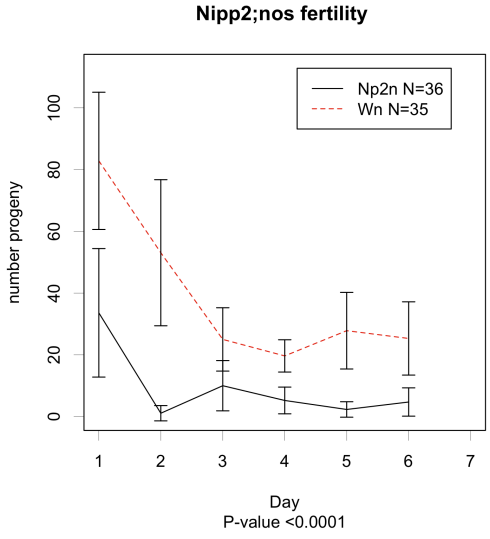
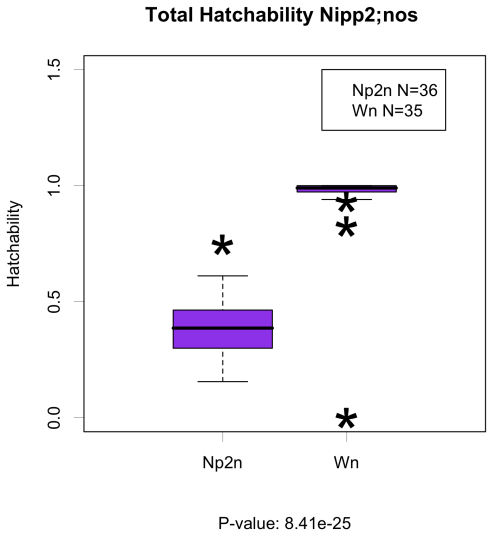
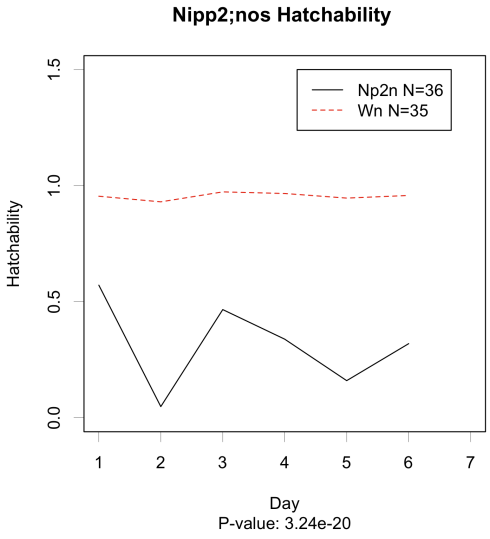
100
200
300
400
Np2n
Wn
P-value: 7.97e-22


Nipp2;nos fertility
Np2n N=36
Wn N=35
number progeny
0
20
40
60
80
100
1
2
3
4
5
6
7
Day
P-value <0.0001


Total Hatchability Nipp2;nos
Np2n N=36
Wn N=35
Hatchability
0.0
0.5
1.0
1.5
Np2n
Wn
P-value: 8.41e-25


Nipp2;nos Hatchability
Np2n N=36
Wn N=35
Hatchability
0.0
0.5
1.0
1.5
1
2
3
4
5
6
7
Day
P-value: 3.24e-20

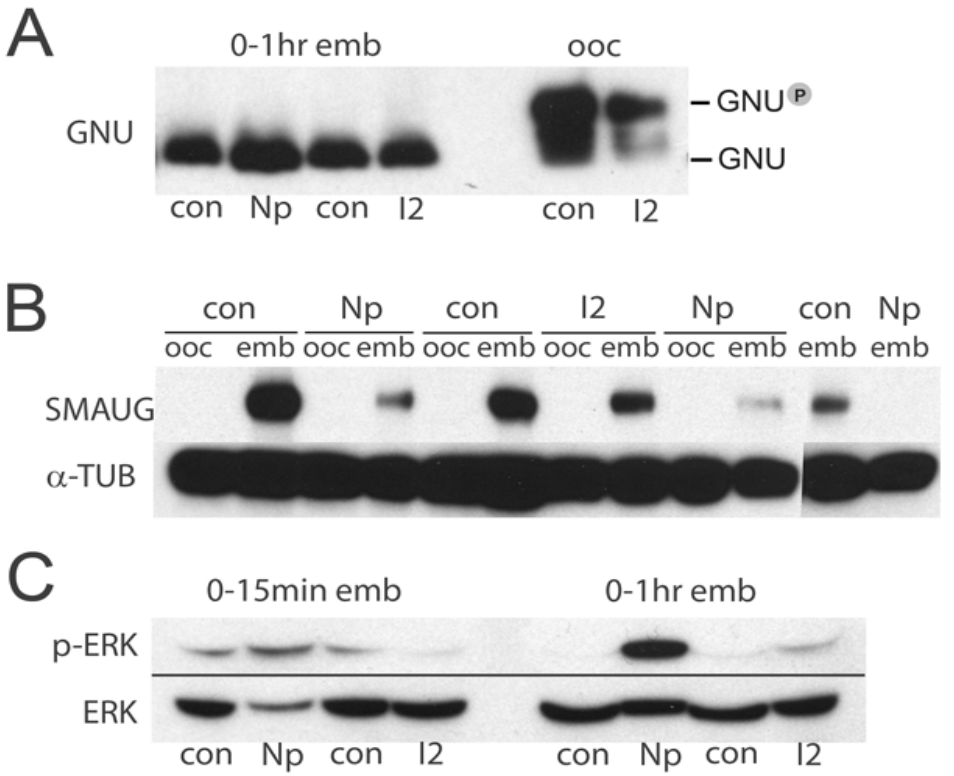
Nipp;MT females and similar to controls (the significant difference was over six days total). The decreased hatchability, which could reflect an egg activation defect or later embryogenesis defects, was significant with both drivers.

To test directly if PP1 inhibitors affected activation, I assayed several activation events. In embryos laid by *UASp-I2Dm; maternal tubulin-Gal4* and *UASp-Nipp; maternal tubulin-Gal4* females, Smaug is translated upon egg activation, although there may be less Smaug protein translated in *UASp-Nipp; maternal tubulin-Gal4* 0-1 hour embryos than controls (Fig 3.10B). In embryos laid by *UASp-I2Dm; maternal tubulin-Gal4* females, levels of phospho-ERK decrease upon egg activation as in wildtype embryos. In embryos laid by *UASp-Nipp; maternal tubulin-Gal4* females, levels of phospho-ERK remain significantly higher than in control embryos (Fig 3.10C). However, this effect is variable and may occur more often in one transgenic line than the other. The trend of high phospho-ERK levels in *UASp-Nipp* embryos is consistent with reduced or slower ERK dephosphorylation upon activation. At the earlier 0-15minute embryo timepoint, two of two *UASp-Nipp* samples have shown increased phospho-ERK, while at the later 0-1hr timepoint, which is a less sensitive assay, two out of four *UASp-Nipp* samples have increased levels of phospho-ERK. The aspect of egg activation most clearly related to Ser/Thr dephosphorylation is the dephosphorylation of GNU upon egg activation. However, the dephosphorylation of GNU occurs normally in embryos laid by *UASp-I2Dm; maternal tubulin* and *UAS-Nipp; maternal tubulin* females (Fig 3.10A).

The effects of these inhibitors appear dosage-dependent, because the stronger *maternal tubulin-Gal4* driver causes stronger phenotypes than *nanos-Gal4*, and the line variation could be explained by variable expression levels based on number of gene insertion copies or transcriptional accessibility of the chromatin where the transgene randomly inserted. Dosage-dependence should be expected, since these

Figure 3.10 When over-expressed in the germline, the Nipp inhibitor of PP1 phosphatases causes elevated levels of phospho-ERK in embryos.

Western blot analysis was performed on total protein extracts of mature ovarian oocytes, 0-15 min and 0-1hr embryos from females driving expression of the indicated UAS-transgene under the control of *Gal-4 maternal tubulin* (except the 3rd to last pair of samples in (B), lanes 9&10, is Np;nos) (A) Anti-ERK was used as a loading control while anti-phospho-ERK was used to detect the active form of ERK. Phospho-ERK is low in 0-15 min and even lower in 0-1hr control embryos, but in embryos laid by Nipp (Np) transgenic females, the level of phospho-ERK (relative to total ERK loading) is high at both time-points. Embryos laid by Inhibitor-2 (I2) females have low phospho-ERK levels relative to total ERK loading, similar to wildtype. Western blots shown are representative of 2 independent replicates with multiple lines each; the level of phospho-ERK varies but shows an overall trend of Nipp-dependent increase. (B) Anti-SMAUG was used to detect SMAUG protein and Anti-α-tubulin antibody was used as a loading control. SMAUG protein is translated (at apparently low levels) in embryos laid by Nipp (Np) transgenic females, and translated at close to normal levels in embryos laid by Inhibitor-2 (I2) transgenic females.


A
0-1hr emb
ooc
GNU
GNU P
GNU
con Np con I2
con I2
B
con Np con I2 Np con Np
ooc emb ooc emb ooc emb ooc emb ooc emb emb emb
SMAUG
α-TUB
C
0-15min emb
0-1hr emb
p-ERK
ERK
con Np con I2
con Np con I2

inhibitors are normally present in many tissues at low levels, and their over-expression by the UAS-Gal4 system is what creates a phenotype. To verify that different lines and drivers are causing different levels of expression, in the future we will assay mRNA levels of *UASp-Nipp* and *UASp-I2Dm* from embryos with expression driven by *nanos-GAL4* or *maternal tubulin-GAL4* by RT-PCR with primers in the UAS sequence and in the gene. Different levels of inhibitor overexpression might be necessary to inhibit different aspects of egg activation. Within the presumed range of expression levels I am assaying, there is no effect on GNU dephosphorylation, although Inhibitor-2 and chemical PP1 inhibitors prevent GNU dephosphorylation *in vitro* (Renault et al. 2003). It is possible that higher expression levels would cause inhibition of GNU dephosphorylation. Although PP1 phosphatases are not thought to directly dephosphorylate MAPKs, my data suggest they may have a role in MAPK dephosphorylation upon egg activation acting through as-yet unknown pathways. PP1 phosphatases may also be involved in the translational regulatory pathways that increase Smaug translation upon egg activation, based on the subtle effect Nipp has on levels of Smaug translation.

Conclusions:

I have begun to identify and/or rule out connections between the proteins that are dephosphorylated upon egg activation, YA, GNU, and MAPKs, and other molecules and aspects of egg activation. In embryos, YA dephosphorylation is downstream of the activation genes *cortex*, *sarah*, and *prage*. In oocytes, YA's phosphorylation is not attributable to ERK alone or p38/JNK together without ERK. Mass spectrometry of YA shows that it is phosphorylated on two MAPK sites (T443 and S674) in both oocytes and embryos, and of the two sites with oocyte-specific phosphorylation (S158 and S392), one is a putative GSK3 target. The at least eleven total phosphorylation sites of YA are likely to be targets of different kinases. GNU dephosphorylation upon

egg activation is downstream of *cortex* and *sarah*, but upstream or independent of *prage*. Neither inhibition of PP1 phosphatases by Nipp and Inhibitor-2 nor knockout of MKP3 in the germline hinders GNU dephosphorylation upon egg activation, nor does the presence of constitutively active ERK, although MAPKs and PP1 phosphatases are known to regulate GNU phosphorylation (Renault et al. 2003; Zhang et al. 2004). However, although active ERK is not sufficient to keep GNU phosphorylated in embryos, my preliminary results suggest that ERK, but not p38 and JNK, is necessary for GNU phosphorylation in oocytes. Knockout of MKP3 in the germline causes zero hatchability due to arrest after fertilization but before the first mitosis. UAS-Gal4 driven expression of PP1 phosphatase inhibitors or constitutively active ERK causes various degrees of reduced hatchability. Reduced hatchability is characteristic of egg activation genes. However, the role of MKP3 might be after activation, since SMG translation and ERK dephosphorylation (as well as GNU dephosphorylation) occur normally in MKP3 knockout clones, and the cell cycle arrest of MKP3 knockout clone embryos is apparently after completion of meiosis. Expressing the PP1 inhibitor Nipp in eggs disrupts subsequent ERK dephosphorylation and SMG translation levels, but constitutively active ERK (rl^{Sem}) has no effect on SMG translation. PP1 phosphatases may be involved in phospho-signaling upstream of SMG translation and ERK dephosphorylation.

CHAPTER FOUR

THE WISPY POLY-A POLYMERASE REGULATES THE PHOSPHOPROTEINS ERK, JNK, YA AND GNU DURING OOCYTE MATURATION[2]

4.1 Introduction

One key aspect of egg activation is the cytoplasmic polyadenylation of certain maternal mRNAs to permit or enhance their translation and improve their stability. Mutations in the *wispy (wisp)* maternal effect gene of Drosophila, which encodes a cytoplasmic poly(A) polymerase (PAP) in the GLD-2 family, block development during the egg-to-embryo transition (Brent et al. 2000; Cui et al. 2008; Benoit et al. 2008). WISP activity is required for poly(A) tail elongation of *bicoid, Toll,* and *torso* mRNAs upon egg activation. In Drosophila, WISP and Smaug (SMG) have previously been reported to be required to trigger the destabilization of maternal mRNAs during egg activation (Tadros et al. 2007). SMG, the major regulator of this activity, is still translated in activated eggs from *wisp* mutant mothers, indicating that WISP does not regulate mRNA stability by controlling the translation of *smg* mRNA.

wisp maternal effect mutations lead to very early developmental arrest prior to embryonic mitosis. Eggs lacking maternal WISP function are capable of completing female meiosis to form decondensed female meiotic products. Although the sperm nucleus appears capable of remodeling and forming a male pronucleus in the absence of WISP function, the sperm asters fail to grow and pronuclear migration does not occur. All the nuclei condense their chromosomes and become associated with a

2 Part of this chapter is published as: Cui J, Sackton KL, Horner VL, Kumar KE, Wolfner MF. (2008) Genetics 178(4):2017-29. The Introduction, Materials and Methods, and "Levels of some active (phospho-) MAPKs are decreased in *wisp* mutant oocytes" section of the Results and Discussion (Figure 4.1) are modified from the paper, and the work of Jun Cui (Figures 4.1C, 4.5A) is acknowledged and identified in the text.

spindle structure, but then arrest at this point. These data are consistent with *wisp*'s being essential for some aspect of the tubulin cytoskeleton in oocytes and embryos.

Because WISP protein is present both in ovaries and in embryos after egg activation (Cui et al. 2008), and because *wisp* mutant oocytes have a defect during the meiosis I arrest prior to egg activation (Brent et al. 2000), we considered the possibility that WISP might have functions in mature oocytes immediately before egg activation. In Drosophila oocytes, levels of active (that is, phosphorylated) MAPKs are high, but upon egg activation (independent of fertilization), the amounts of the active forms of the MAPKs decrease although the total amounts of the MAPK proteins do not change (Sackton et al. 2007, Chapter 2). Three classes of MAPK [extracellular signal-related kinase (ERK), Jun N-terminal kinase (JNK), and p38] are present in Drosophila, as in other organisms. The change in MAPK activity between mature oocytes and activated eggs suggests that MAPK-signaling cascades have a necessary function in oocytes and lack of MAPK-signaling could be important upon egg activation.

We find that WISP function is needed during oogenesis to regulate the poly(A) tail length of *dmos* mRNA during oocyte maturation and to maintain a high level of active (phospho-) mitogen-activated protein kinases (MAPKs) and an active (phospho-) MAPK kinase (MEK). WISP function is also required for normal levels of the proteins YA and GNU in oocytes, although the subsequent presence of these proteins in embryos is not *wisp*-dependent. Both GNU and YA function after egg activation in regulating the early embryonic cell cycles (Freeman et al. 1987; Lin et al. 1991 and Chapter 5). YA and GNU are both present in oocytes in phosphorylated forms, but are dephosphorylated upon egg activation. YA 's dephosphorylation correlates with its developmentally regulated change in subcellular localization from cytoplasmic in oocytes to nuclear in embryos (Yu et al. 1999), and YA function is

required for the first embryonic mitosis (Lin et al. 1991). The role of GNU's developmentally regulated dephosphorylation is not fully understood, but GNU functions as part of the PanGu kinase complex, which regulates embryonic S-M cycles (Lee et al. 2003). I have detected YA and GNU proteins by western blots in stages 8-12 of oogenesis, but not earlier, which is consistent with previous fluorescence microscopy data (Renault et al. 2003)(N. Buehner unpublished results). YA and GNU protein levels increase in mature oocytes.

4.2 Materials and Methods

Drosophila stocks:

Oregon-R was used as the wild type stock. *wisp*^{41}/FM6, *wisp*^{248}/FM6 and *wisp*^{249}/FM6 (Tadros et al. 2003) were kindly provided by W. Tadros and H. Lipshitz (Hospital for Sick Children, University of Toronto, Canada). *sra*^{687}/TM6 and *Df(3R)sbd*^{45}*, mwh*^{1} *e*^{1} /TM6 were previously described by (Horner et al. 2006). *Df(1)RA47*/FM7c -used for making *wisp*^{41} and *wisp*^{248} hemizygotes was from the Bloomington *Drosophila* Stock Center.

Embryo collection, fixation and staining:

Laid eggs were collected as previously described (Horner et al. 2006). Three to five-day-old virgin females were mated either to wild-type Oregon-R males to produce fertilized eggs (embryos) or to spermless males [the sons of *tud*^{1} *bw sp* mothers (Boswell et al. 1985)]to generate unfertilized activated eggs. Fertilized and unfertilized eggs were collected on grape juice plates for the desired period of time and washed off the plates with egg wash buffer (0.4% NaCl, 0.2% Triton X-100). Hereafter, “0- to 1-hr embryos” will refer to fertilized eggs collected 0- to 1-hr post egg deposition, and other collection lengths will be described in similar fashion. “Eggs” will refer to eggs laid by females mated to wild-type males, which may be

fertilized or unfertilized, and "unfertilized eggs" will refer to eggs laid by females mated to spermless males (described above), unless otherwise indicated. For immunofluorescence analysis, collected eggs were dechorionated in 50% bleach for 2 min, rinsed thoroughly with water, then permeabilized with heptane and fixed immediately in cold methanol. Fixed eggs were washed in 1 X PBST (137 mM NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 0.1% Triton X-100) and incubated at 4° overnight with a 1:200 dilution of a mouse anti-α-tubulin antibody (Sigma, St. Louis, MO) in 1 X PBST. RNaseA (Roche Applied Science, Indianapolis, IN) was added to a final concentration of 5 μg/ml. Secondary antibody [Alexa Fluor 488-conjugated goat anti-mouse antibody (Invitrogen, Carlsbad, CA) at a dilution of 1:200] was then added for 2 hr at room temperature. Propidium iodide (Invitrogen) was added at a concentration of 10 μg/ml to stain DNA. Samples were either mounted in 75% glycerol containing 940 mM *n*-propyl gallate or washed with methanol and mounted in benzyl benzoate:benzyl alcohol (2:1). Staining in fertilized and unfertilized eggs was analyzed using confocal microscopy [Leica TCS SP2 system equipped with an argon-krypton laser and coupled to a Leica DMRBE microscope (Leica Microsystems, Germany)]. Leica software was used to collect images and, where appropriate, to project multiple optical sections into a single image and to overlay images.

RNA extraction and RT-PCR:

Total RNA was extracted from mature oocytes using TRIzol (Invitrogen) and reverse transcribed using SuperScript II Reverse Transcriptase (Invitrogen). PCR reactions were performed with GoTaq reaction buffer (Promega, Madison, WI). Primer sequences were as follows: Ya Fwd 5'ATGTCGTTTTCCAATGTCCTAATC, Ya Rev 5'-ATGGCGATGCTCGATGATAC, gnu Fwd 5'-AGCGCTACAATCGCGTCTAT, gnu Rev 5'-GGCATTCCAAAGAGTCCGTA. The transcript for ribosomal protein RPL32 (Fiumera et al. 2005), amplified using

primers 5'-CCGCTTCAAGGGACAGTATC and 5'-GACAATCTCCTTGCGCTTCT, was used as a control for the quality and quantity of cDNAs used for RT-PCR.

Western blot analysis:

Samples from ovarian oocytes and 0- to 15-min or 0- to 2-, 2- to 4-, 4-to 6-, 6- to 24-hr embryos were prepared for Western blotting and processed as in (Sackton et al. 2007). Depending on sample abundance and antibody strength, ~20 to ~70 μg total soluble protein (TSP) were loaded per well. A polyclonal anti-WISP antibody was generated by J. Cui by methods described in (Ravi Ram et al. 2005). Briefly, a glutathione-S-Transferase (GST) fusion of the carboxy-terminal 411 amino acids of the predicted WISP protein was purified from *Escherichia coli* cells and used to immunize rabbits (Cocalico Biologicals Inc., Reamstown, PA). Serum was first run through a Sepharose 4B column (Sigma) coupled with GST protein and then affinity purified with the GST-WISP fusion protein. The purified antibody was used at 1:2,000 dilution to probe Western blots. Anti-α-tubulin antibody (Sigma, catalog #T5168) was diluted 1:10,000. Anti-MAPK antibodies were used as previously described (Sackton et al. 2007). Anti-phospho-MEK (Sigma, catalog #M7683)was diluted 1:1000. Guinea-pig anti-GNU [gift of T. Orr-Weaver, (Lee et al. 2003)] was diluted 1:5000. Anti-YA (Liu et al. 1995) was diluted 1:1,000.

Poly(A) tail assay:

Oocytes were dissected from 3-4 day old virgin control and virgin *wisp* mutant females. 0-1 hr embryos were collected as described above and aged at room temperature for 1 or 2 hours to get 1-2 or 2-3 hr embryos, respectively. Total RNA was extracted using TRIzol (Invitrogen), and 1μg total RNA from each sample was used for poly(A) tail (PAT) assay. PCR-based PAT assays were performed as described in (Sallés et al. 1994). Briefly, total RNA from each sample was incubated in the presence of 20 ng 5'-phosphorylated oligonucleotide $p(dT)_{18}$ to saturate the poly(A)

tails of the mRNAs. The $p(dT)_{18}$ was then ligated together in the presence of 10 units (Weiss) of T4 DNA ligase (Fermentas, Glen Burnie, MD) to generate a complementary copy of the poly(A) tail. Two hundred nanograms of oligo$(dT)_{12}$-anchor was added and ligated to the 5'-end of the poly(T) strand. The mRNAs were then reverse transcribed using SuperScript II Reverse Transcriptase (Invitrogen) to synthesize the PAT cDNAs. PCR was performed on the PAT cDNAs using a gene-specific primer and the oligo$(dT)_{12}$-anchor to test the length of the poly(A) tail of a specific mRNA. Sequences of primers specific for *bicoid*, *Toll*, and *torso* and the sequence of the oligo$(dT)_{12}$-anchor were described by (Sallés et al. 1994). The sequence of our primer specific for *dmos* is 5'-GCTGAAGCATGAGCTGGAATTC. PCR products from the PAT assays were run on 2% agarose gels or 8% acrylamide gels. Gels were stained with 0.5 μg/ml ethidium bromide.

4.3 Results and Discussion

Levels of some active (phospho-) MAPKs are decreased in *wisp* mutant oocytes:

To determine if *wisp* affects MAPK activity at or before egg activation, I examined the amount of phospho-MAPKs in *wisp* mutant oocytes and embryos. In $wisp^{41}$/+ controls (Figure 4.1, panel A, left), the level of phospho-ERK is high in mature (stage 14) oocytes and decreases upon egg activation as reported previously for wild type (Sackton et al. 2007, Chapter 2). In contrast, the level of phospho-ERK in *wisp* mutant mature oocytes is much lower than in control mature oocytes (Figure 4.1A, right). Similarly, stage 14 *wisp* oocytes also contain an unusually low level of phospho-JNK relative to controls, although their levels of phospho-p38 are normal (Figure 4.1B). Upon egg activation, the levels of phospho-ERK and phospho-JNK in *wisp* embryos are very low or undetectable, as in wild-type embryos (Figure 4.1, A and B). These results suggest that *wisp* plays a role in regulating MAP kinase activity

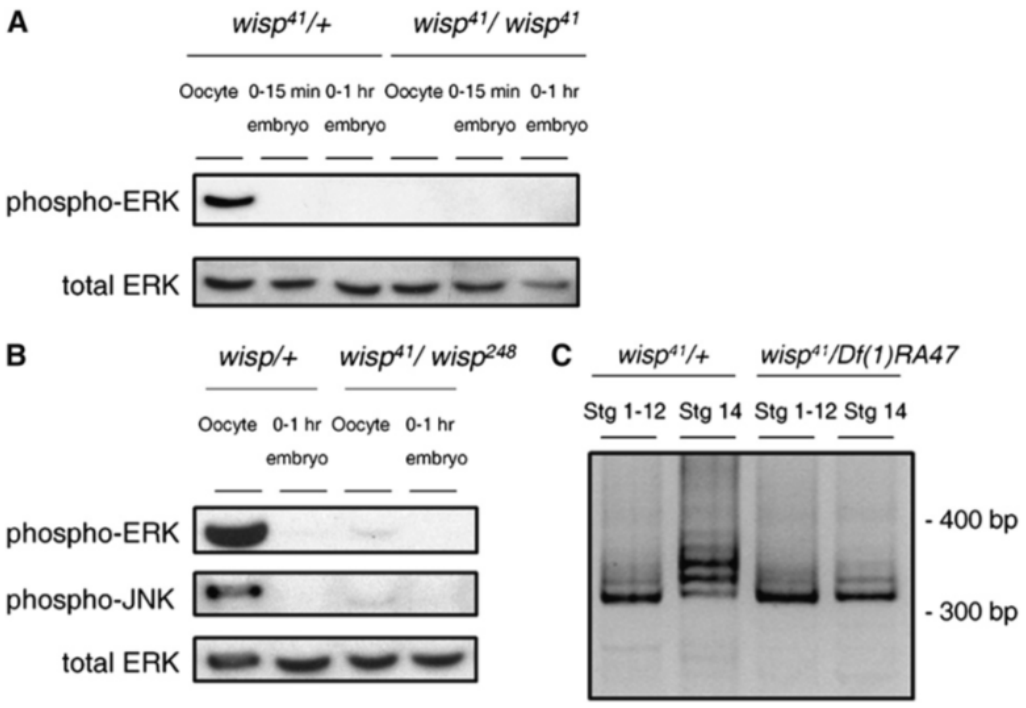
during oogenesis.

Because total ERK protein levels are normal in *wisp* mature oocytes, WISP regulates the activation of this kinase, not its translation. WISP could regulate translation of an upstream activating kinase or kinases in oocytes, so that these kinase(s) in turn can regulate the phosphorylation and activity of ERK and JNK. Studies in Xenopus have shown that CPEB-mediated polyadenylation regulates the translation of *mos* mRNA during oocyte maturation (Sheets et al. 1995; Stebbins-Boaz et al. 1996), and that MOS phosphorylates MAPK/ERK kinase (MEK), a kinase that then phosphorylates MAPKs in mature oocytes (Maller et al. 2002). Decreased levels of phospho-ERK and phospho-MEK were observed in oocytes from *dmos* mutants in Drosophila (Ivanovska et al. 2004). MOS has also been shown to activate JNK in Xenopus eggs and embryos (Bagowski et al. 2001; Mood et al. 2004). Preliminarily results from *dmos* mutants suggest both ERK and JNK are phosphorylated downstream of MEK in Drosophila oocytes (N. Buehner, preliminarily data) as suggested from observations in other species.

The decreased levels of phospho-ERK and phospho-JNK in *wisp*-deficient Drosophila mature oocytes could be attributable to upstream regulation by DMOS. We explored the possibility that *dmos* transcripts' poly(A) tails could be extended during oocyte maturation, as is the case for Xenopus *mos*, and for Drosophila *cortex* and *cyclin B* transcripts (Benoit et al. 2005; Pesin et al. 2007), and that *wisp* could mediate this, by analogy to the requirement for xGLD-2 for CPEB-mediated polyadenylation during Xenopus oogenesis (Barnard et al. 2004). Jun Cui used PAT assays to examine *dmos* mRNA's poly(A) tail length in immature (stage 1-12) and mature (stage14) oocytes from control females or *wisp* mutant females. In the controls (Figure 4.1C, two lanes on left), *dmos* transcripts have shorter poly(A) tails in stage 1-12 (immature) oocytes; their poly(A) tails are longer by ~40 nt in stage 14 (mature) oocytes. Thus,

Figure 4.1 Levels of phospho-ERK and phospho-JNK, and lengths of *dmos* poly(A) tails, are decreased in *wisp* mutant oocytes.

(A) Western blot analysis was performed on total protein extracts of mature (stage 14) ovarian oocytes, 0-15-min or 0-1-hr embryos from $wisp^{41}$/ $wisp^{41}$ or $wisp^{41}$/+ females. Anti-ERK was used to detect total ERK protein while anti-phospho-ERK was used to detect the active form of ERK. (B) Anti-phospho-JNK was used to detect the active form of JNK, and anti-phospho-p38 was used to detect the active form of p38, in total protein extracts (40-70μg TSP/well) of mature oocytes or 0- to 1-hr embryos from $wisp^{41}$/ $wisp^{248}$ or heterozygous control females. Anti-ERK was used here as a loading control. Levels of phospho-ERK and phospho-JNK, but not phospho-p38, are low in wisp mutant oocytes. (C) Stage 1-12 and stage 14 oocytes were hand dissected from $wisp^{41}$/+ control and $wisp^{41}$/*Df(1)RA47* mutant females. Jun Cui performed PAT assays to examine the poly(A) tail length of dmos mRNA. PAT assay products were separated by DNA PAGE. Molecular size markers are shown on the right. In the controls (two lanes on left), the length of *dmos* mRNA poly(A) tail is ~40 nt longer in stage 14 oocytes than in stage 1-12 (two lanes on left), indicating extension of *dmos* mRNA poly(A) tails during oocyte maturation. In contrast, this increase is not seen in *wisp* mutant oocytes (two lanes on right). Western blots shown are representative of 4 independent replicates.


A
wisp41/+
wisp41/ wisp41
Oocyte
0-15 min embryo
0-1 hr embryo
Oocyte
0-15 min embryo
0-1 hr embryo
phospho-ERK
total ERK
B
wisp/+
wisp41/ wisp248
Oocyte
0-1 hr embryo
Oocyte
0-1 hr embryo
phospho-ERK
phospho-JNK
total ERK
C
wisp41/+
wisp41/Df(1)RA47
Stg 1-12
Stg 14
Stg 1-12
Stg 14
- 400 bp
- 300 bp

dmos mRNA poly(A) tails lengthen during oocyte maturation in Drosophila (as in Xenopus). In contrast to the situation in controls, the length of the poly(A) tails of *dmos* mRNA in mature oocytes of $wisp^{41}$ mutant females is not significantly different from that in immature oocytes of these females (Figure 4.1C, two lanes on right). Therefore, *wisp* function is needed for poly(A) tail extension on *dmos* mRNA during oocyte maturation.

The shorter poly(A) tail in mature *wisp* oocytes could lead to a failure or inefficiency of *dmos* translation that would in turn explain the decreased levels of phospho-ERK and phospho-JNK. Unfortunately we are unable to examine DMOS protein levels in Drosophila because there is no suitably specific antibody available. However, I find that phospho-MEK levels are low in *wisp* mutant mature oocytes (Figure 4.2), supporting the hypothesis that MAPK phosphorylation is regulated through the upstream kinases MEK and DMOS during Drosophila oocyte maturation, and that *wisp* controls this by controlling the polyadenylation of *dmos* mRNA (at least) during egg maturation.

Although levels of phospho-ERK and phospho-JNK are low or undetectable in *wisp* mutant mature oocytes, meiosis can still complete upon activation of those eggs. This result is consistent with a previous report that oocytes from *dmos* mutants have decreased levels of phospho-ERK yet they can complete meiosis (Ivanovska et al. 2004). Although MAPK activity downstream of DMOS is not necessary for completion of meiosis in Drosophila, the regulation of MAPKs or other genes by WISP may be important for other aspects of late oocyte development or for making the oocyte competent to activate. For instance, ERK has at least nine different roles in C.elegans oocytes (Lee et al. 2007).

Levels of YA and GNU are decreased in *wisp* mutant oocytes:

GNU and YA phosphoproteins are MAPK targets, and their phosphorylation

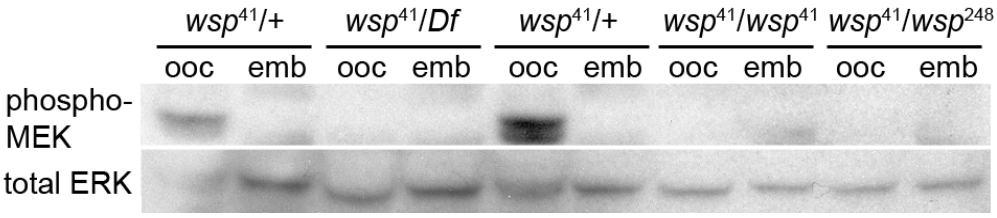


Figure 4.2 Levels of phospho-MEK are decreased in *wisp* mutant oocytes.

Western blot analysis was performed on total protein extracts (30-40 μg TSP/well) of mature (stage 14) ovarian oocytes and 0-1-hr embryos from *wisp*^{41}/ *wisp*^{41}, *wisp*^{41}/ *Df(1)RA47*, *wisp*^{41}/ *wisp*^{248}, or *wisp*^{41}/+ females. Anti-ERK was used to detect total ERK protein while anti-phospho-MEK was used to detect the active form of MEK. A faint cross-reactive band is visible below phospho-MEK in most lanes. Western blots shown are representative of 3 independent replicates.

correlates with MAPK activity, because they are phosphorylated in oocytes when MAPKs are active, and dephosphorylated upon egg activation, when MAPK activity decreases (Renault et al. 2003; Yu et al. 1999). In *wisp* mutant mature oocytes dissected from ovaries, levels of YA and GNU protein are greatly reduced, and the trace amounts visible are the dephosphorylated forms which are normally only seen after egg activation (Figure 4.3). The phosphorylated forms migrate more slowly and therefore have higher apparent molecular weights, while the dephosphorylated forms migrate faster and have lower apparent molecular weights. The reduced levels of these proteins in *wisp* mutant mature oocytes could be due to protein instability attributable to improper phosphorylation state, protein instability due to lack of a stabilizing protein interactor, or it could reflect lower levels or translation efficiency of *Ya* and *gnu* RNA. For instance, if *gnu* and *Ya* mRNA translation is regulated by polyadenlyation, the levels of these proteins could be low because the transcripts are present with insufficiently long poly(A) tails. An additional or alternative form of transcript regulation by polyadenylation would be if *gnu* and *Ya* mRNA stability is increased by polyadenylation, so that with short poly(A) tails, these transcripts are degraded. Abnormal phosphorylation state is not sufficient to cause YA protein instability in the case of YA phosphorylation on MAPK sites, since transgenic mutant protein with all the S/T of MAPK sites changed to A is present at normal levels in oocytes (N. Buehner, unpublished results, see Chapter 3).

To determine which of these regulatory mechanisms are acting on *Ya* and *gnu* in oogenesis we assayed protein and mRNA levels at various stages of wild-type oogenesis. Previous fluorescence microscopy studies of YA and GNU expression found detectable levels of GNU-GFP in stage 11 and older egg chambers (Renault et al. 2003) and detected YA in stage 9 and older by immunostaining (N. Buehner unpublished results). Consistent with the immunofluorescence data, my western blots

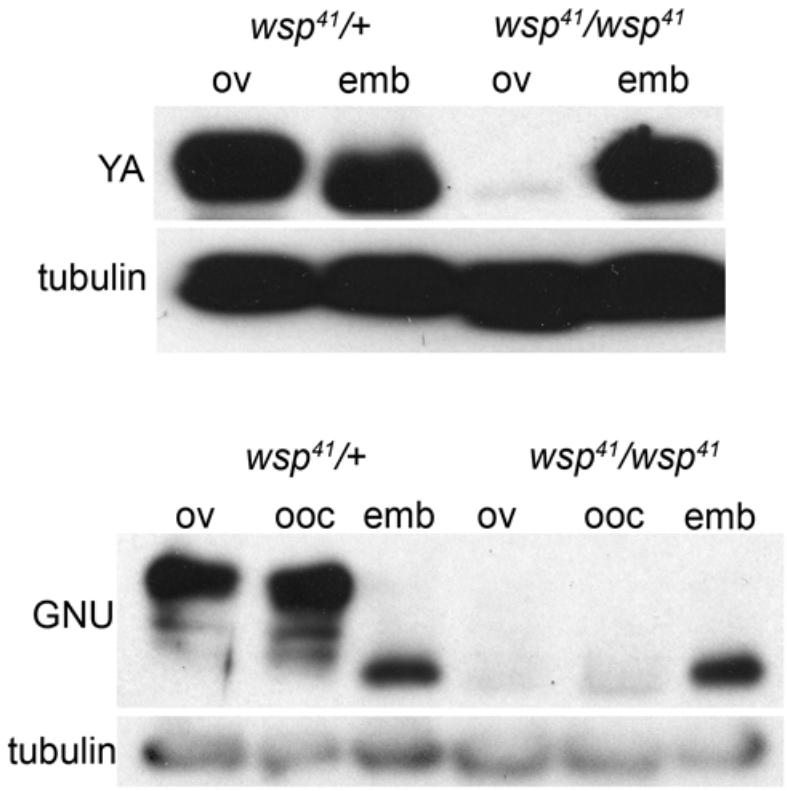


Figure 4.3 Levels of YA and GNU are decreased in *wisp* mutant oocytes, but normal in *wisp* mutant embryos.

Western blot analysis was performed on total protein extracts (~40 μg TSP/well) of whole ovaries, mature (stage 14) ovarian oocytes, and 0-1-hr embryos from *wisp*^{41}/*wisp*^{41} and *wisp*^{41}/+ females. The phosphorylated (slower migrating) and dephosphorylated (faster migrating) forms of YA and GNU can be distinguished, and are labelled. Anti-α-tubulin was used as a loading control. Western blots shown are representative of 3 independent replicates.

did not detect YA and GNU in oogenesis stages 1-7, although WISP is detected at low levels in these stages (Figure 4.4). Although very low levels of YA and GNU are detected in stages 8-12, their protein levels increase dramatically in stage 14 (mature) oocytes. Thus translation or stability of both proteins increases dramatically during oocyte maturation.

Because YA and GNU protein levels are increased upon oocyte maturation, and because wisp affects polyadenylation during maturation (Cui et al. 2008) Jun Cui performed PAT assays of poly(A) tail length for *Ya* and *gnu* mRNAs during oogenesis. He found that the poly(A) tails of *Ya* and *gnu* mRNAs increase in length upon oocyte maturation in a *wisp*-dependent manner (Figure 4.5A). Upon oocyte maturation in *wisp* mutant oocytes (stage 12 to stage 14), when *Ya* and *gnu* mRNAs' poly(A) tails are not lengthened, YA and GNU protein levels do not increase above the very low levels detected prior to oocyte maturation. Therefore the increase in YA and GNU protein levels upon oocyte maturation is due to *wisp*-dependent polyadenylation of *Ya* and *gnu* mRNAs.

To determine whether polyadenylation of *Ya* and *gnu* mRNAs increases transcript stability, translation efficiency, or both, I performed semi-quantitative RT-PCR from *Ya* and *gnu* mRNAs (using ~100 base pair regions). I compared the PCR product intensities of *Ya* or *gnu* to a similar-sized PCR product of a housekeeping gene, rpl32. This comparison provided a semi-quantitative “normalization”. My preliminary results (one experiment with three independent biological samples) show a trend of lower *Ya* mRNA levels in *wisp* mutant mature oocytes (“normalized” to rpl32) compared to levels in control mature oocytes (also “normalized” to rpl32) (Fig 4.5B). The rpl32 control gene is detected at lower levels in mutant than control, suggesting uneven loading of total RNA into the RT-PCR reactions. The *gnu* mRNA levels (when “normalized” to rpl32) do not seem to differ between *wisp* mutant and

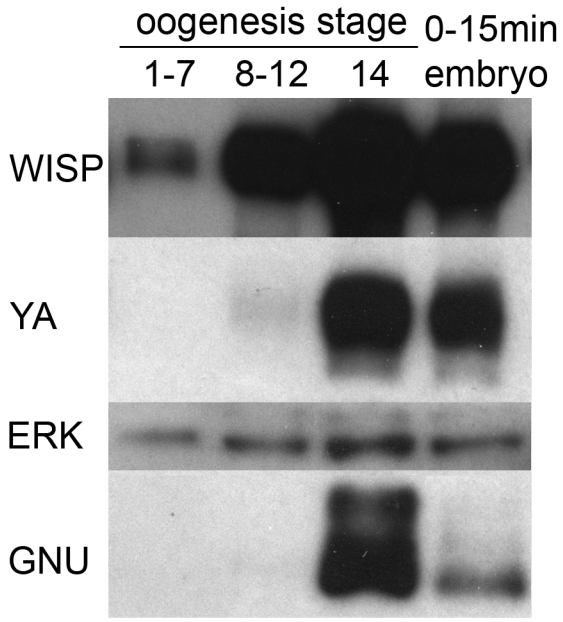


Figure 4.4 Levels of WISP protein increase as oogenesis progresses, but levels of YA and GNU proteins significantly increase only upon oocyte maturation. Western blot analysis was performed on total protein extracts (~20 μg TSP/well) of oocytes dissected from ovaries and sorted into oogenesis stages 1-7, 8-12, and stage 14 (mature), and total protein extracts of 0-15 min embryos from wild-type (ORP2) females. Anti-ERK was used as a loading control. The phosphorylated (slower migrating) and dephosphorylated (faster migrating) forms of GNU can be distinguished, but the electrophoresis of YA was not run long enough to separate its phospho-forms. Western blots shown are representative of 2 independent replicates.

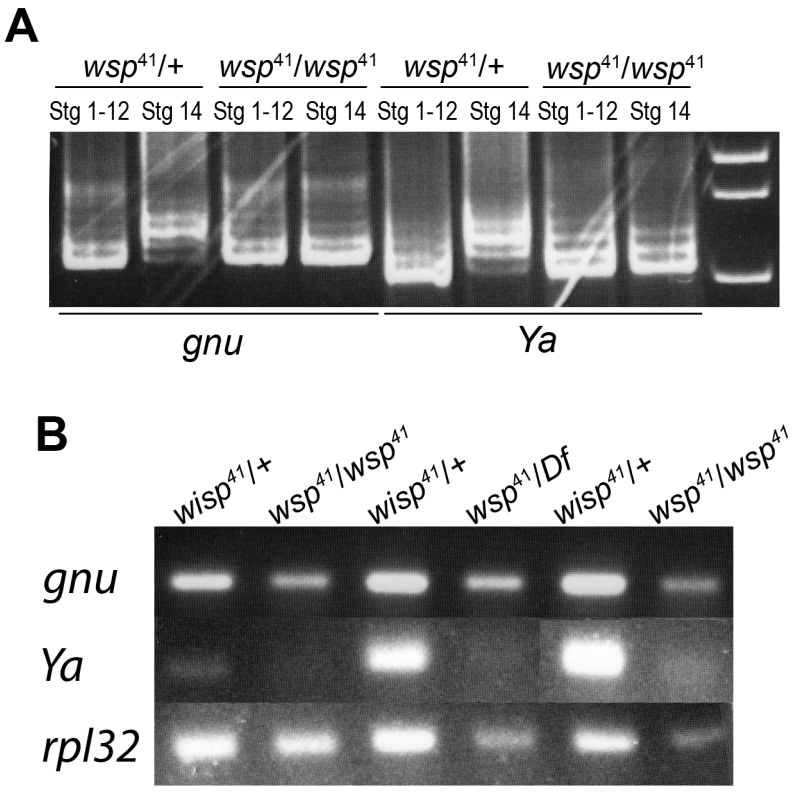


Figure 4.5 Preliminary. *Ya* and *gnu* mRNAs are present in *wisp* mutant mature oocytes, and *Ya* and *gnu* mRNAs are polyadenylated upon oocyte maturation in a *wisp*-dependent manner.

(A) Stage 1-12 and stage 14 oocytes were hand dissected from $wisp^{41}$/+ control and $wisp^{41}$ mutant females. PAT assays were performed to examine the poly(A) tail length of *Ya* and *gnu* mRNA. PAT assay products were separated by DNA PAGE. Molecular size markers are shown on the right. In the controls (two lanes on left for each gene tested), the length of mRNA poly(A) tail is longer in stage 14 oocytes than in stage 1-12, indicating extension of mRNA poly(A) tails during oocyte maturation. In contrast, this increase is not seen in *wisp* mutant oocytes (two lanes on right for each gene). (B) RT-PCR was performed on cDNA made from RNA isolated from stage 14 oocytes of the genotypes $wisp^{41}$/ $wisp^{41}$, $wisp^{41}$/ *Df(1)RA47*, or $wisp^{41}$/+. The ribosomal gene *rpl32* was amplified from the same templates to control for RNA loading.

control mature oocytes. This is consistent with J. Cui's microarray data that *gnu* transcript levels are not significantly different between *wisp* mutant and control samples for either mature oocytes or embryos; there is no *Ya* data from these microarrays (J. Cui, unpublished results). The fact that there are detectable YA and GNU PCR products in the *wisp* mutant mature oocyte RT-PCR reactions suggests that *gnu* mRNAs remain stable in *wisp* mutant oocytes, and *Ya* mRNAs are only somewhat destabilized, but both are translated less efficiently because of shorter than normal poly(A) tails. This is consistent with the detection of normal levels of YA and GNU proteins in *wisp* mutant embryos (Fig 4.3, right-most lanes).

In embryos, *Ya* and *gnu* mRNA poly(A) tails may be elongated by another poly-A polymerase (PAP) after egg activation since the mRNA is still present. This model of different sets of PAPs acting on *Ya* and *gnu* mRNAs during oocyte maturation (wispy) and after egg activation (unknown other PAP and possibly also wispy) is more consistent with known conditions of Drosophila embryogenesis than a model where *Ya* and *gnu* mRNAs are degraded in *wisp* mutant mature oocytes, because the latter model would require transcription of the *Ya* and *gnu* genes after egg activation. Almost no transcription occurs in the first two hours of Drosophila embryo development. During that time most proteins are synthesized exclusively from maternally-loaded mRNAs. This makes a model involving regulation of YA and GNU levels via transcription after egg activation highly unlikely. Consistent with this, my preliminary RT-PCRs show that *Ya* and *gnu* mRNAs are present in *wisp* mutant mature oocytes. The PAT assay of *Ya* and *gnu* mRNAs also detected the presence of both mRNAs in *wisp* mutant mature oocytes, and the RT-PCR was intended to quantify their levels, although the preliminary results are not highly quantifiable because of significant differences in the control gene.

Conclusions:

The *wispy* poly(A) polymerase functions in oocyte maturation to polyadenylate *dmos*, *Ya*, and *gnu* mRNAs. The phosphorylation in mature oocytes of proteins downstream of DMOS kinase activity is *wisp*-dependent. These include MEK, its target ERK, and JNK, although the mature-oocyte-specific function of these kinases is not yet known. Levels of YA and GNU protein increase upon oocyte maturation, perhaps to provision sufficient levels of protein that are inactive due to phosphorylation, but ready to act at the first embryonic mitosis upon their dephosphorylation.

CHAPTER FIVE

THE YA GENE IS NEEDED FOR PROPER NUCLEAR ORGANIZATION TO TRANSITION BETWEEN MEIOSIS AND MITOSIS IN DROSOPHILA[3]

5.1 Introduction

Combining two gametes to form a zygote is a highly regulated developmental transition. The male and female gametes are each specialized cells with characteristics that are required for successful fertilization. For example, the sperm is compact for enhanced mobility, and the oocyte is provisioned with elements necessary to support embryogenesis, but quiescent, waiting for egg activation and fertilization. Mutations of Drosophila genes that regulate the gamete to embryo transition cause parental-effect sterility, since the zygotic genotype is not relevant because it cannot be expressed before the zygotic genome is formed from its haploid parent nuclei. Investigations of parental-effect genes contribute to understanding this transition from meiosis to mitosis.

Since the arrest point of *fs(1)Ya*^{2} mutant embryos is prior to the first mitosis, the *Ya* gene's function could be necessary for the completion of meiosis, subsequent nuclear associations, or regulation of cell cycle state of the nuclei during the first mitotic division. Drosophila parental-effect genes essential at the gamete to embryo transition can have one or more such roles, or can regulate the male pronucleus. Acting first developmentally, genes involved in oocyte activation/completion of meiosis include both the egg activation genes *sarah*, *cortex*, and *prage* discussed in earlier chapters, which arrest during meiosis, in egg activation (Chu et al. 2001;

3 This chapter is being submitted at part of Sackton K.L.*, Lopez J.M.*, Berman C.L., and Wolfner M.F., "The YA gene is needed for proper nuclear organization to transition between meiosis and mitosis in Drosophila"

Horner et al. 2006; Tadros et al. 2003), and genes with general meiotic defects that have a later final arrest point. Several genes that encode microtubule-associated proteins (e.g. *asp*, *klp3A)* are necessary for pronuclear migration; their mutants arrest before the first (gonomeric) mitosis (Riparbelli et al. 2002; Williams et al. 1997). There are also genes (*ssm/hira, chd1*) whose products act to remodel the sperm nucleus to allow it to participate in the first mitosis (Loppin, Bonnefoy, et al. 2005; Konev et al. 2007). Still other genes (*plu, gnu, png*) encode regulators of the maternally-driven cell cycles. These genes' products may act during the first mitosis and/or subsequent maternally-driven embryonic cell cycles (Lee et al. 2003). YA-deficient embryos arrest in the first (gonomeric) mitosis. Three other genes whose mutants have a pre-gonomeric arrest; *wispy*, *polo* (only 2% reach pronuclear apposition), and *nucleosomal histone kinase-1* (NHK1, gonomeric metaphase arrest), also have earlier defects in meiosis (Cui et al. 2008; Ivanovska et al. 2005; Riparbelli et al. 2000). In this chapter, based on known aspects of egg activation and the well-characterized stages of meiotic progression and completion, I have shown that *Ya* functions after meiosis, independent of other aspects of egg activation, during or after the post-meiotic S phase.

Mature Drosophila oocytes are arrested in metaphase of meiosis I, with the spindle parallel to the long axis of the egg (Foe 1993). Ovulation triggers a number of changes that are collectively called egg activation (Heifetz et al. 2001; Horner et al. 2006; Mahowald et al. 1983; Page et al. 1997; Takeo et al. 2006). Vitelline membrane proteins undergo cross-linking, certain maternal RNAs are polyadenylated and translated and others are degraded (Tadros et al. 2003, 2005), several proteins' phosphorylation states change (Renault et al. 2003; Sackton et al. 2007; Yu et al. 1999 and V. Horner and A. Krauchunas, unpublished), and meiosis resumes. The mechanism of egg activation is not completely understood. Although in many

organisms activation requires fertilization (Runft et al. 2002), in Drosophila (Heifetz et al. 2001) and other insects examined (King et al. 1970; von Borstel 1957; Went et al. 1974), egg activation is independent of fertilization, although they usually occur at close to the same time. Upon activation, Drosophila oocytes complete meiosis rapidly without apparent chromatin decondensation or cytokinesis, resulting in four haploid nuclei located near the membrane and aligned perpendicular to the long axis of the egg (Endow et al. 1998; Huettner 1924).

Following completion of meiosis, the chromosomes of all four meiotic products decondense. They appear morphologically similar to interphase nuclei, as judged by DNA staining (Foe 1993). The innermost meiotic product usually becomes the maternal pronucleus. The remaining three meiotic products (polar body nuclei) associate near the surface of the egg (Foe 1993; Sonnenblick 1950). In unfertilized, activated eggs, all four meiotic products synchronously replicate their DNA once, then condense their chromosomes [B. Loppin personal communication, (Foe 1993)]. They then associate until all the nuclei have become part of a polar body, which appears as a rosette-shaped array of condensed chromosomes (Doane 1960; Liu et al. 1995). In fertilized eggs, the sperm nucleus undergoes reorganization to become the paternal pronucleus. Its chromatin decondenses and recruits maternally provided proteins, such as histones and YA (Liu et al. 1997; Lopez et al. 1994; Loppin, Bonnefoy, et al. 2005). The maternal and paternal pronuclei migrate towards each other and closely appose for the first (gonomeric) mitotic division. This migration requires microtubules nucleated at the male pronucleus and microtubule associated proteins such as Ncd (non-claret disjunctional), α-tubulin 67C, KLP3A (kinesin-like protein at 3A), and Asp (abnormal spindle protein) (Endow et al. 1998; Riparbelli et al. 2002; Skold et al. 2005; Williams et al. 1997). In embryos, the first S phase likely occurs just prior to or concurrent with apposition, based on staining with the S-phase marker

PCNA (Proliferating Cell Nuclear Antigen) (Loppin et al. 2005). The two pronuclear genomes divide on a shared spindle. Because remnants of the nuclear envelope remain around the pronuclei, the genomes remain separate until telophase, when they mix resulting in two zygotic nuclei (Huettner 1924; Sonnenblick 1950). For genes required for the paternal genome to participate in zygotic mitosis, the mutant phenotype is characteristically that the female pronucleus proceeds through one or more mitotic divisions, producing a nonviable haploid embryo, while the male nucleus either does not reorganize or appose with the female pronucleus (Fitch et al. 1998; Ohsako et al. 2003; Wilson et al. 2006), or it apposes and a mitotic spindle forms, but the paternally-derived chromosomes do not participate in mitosis (Loppin et al. 2001; Loppin, Bonnefoy, et al. 2005; Loppin, Lepetit, et al. 2005). For example, HIRA is a histone H3.3 chaperone that is required for incorporation of maternally-provided histone H3.3 into the male pronucleus, and *Hira/ssm* mutant males sire haploid (inviable) embryos (Loppin, Bonnefoy, et al. 2005). The chromatin-remodelling complex CHD1 interacts with HIRA to facilitate assembly of H3.3-containing nucleosomes (Konev et al. 2007).

After the gonomeric division, thirteen rapid mitotic divisions occur without cytokinesis. During these cycles, S and M phases are tightly coupled. The Pan Gu kinase, which requires the activities of the genes *plu, gnu,* and *png, is* necessary for this aspect of cell cycle regulation. Loss of function of any of these three maternal-effect genes results in embryos with repeated rounds of replication (S phase) without mitosis, resulting in giant nuclei (Lee et al. 2003). These three genes and *Ya* appear to function specifically in the maternally driven cell cycles of the early embryo.

There are between one and five nuclei in Ya^2 (YA-deficient) embryos, and between one and four nuclei in YA-deficient unfertilized eggs (Lin and Wolfner 1991). The numbers suggested that the nuclei in unfertilized eggs were derivatives of female meiosis and the extra nucleus in embryos was from the sperm (Since *Ya* eggs are

known to be fertilized), but whether *Ya* eggs had the ability to properly complete meiosis and the nature of any nuclear associations were unknown. Jacquie Lopez used fluorescent in situ hybridization (FISH) to examine the nuclei in Ya^2 mutant eggs and zygotes (J. Lopez, PhD thesis, 1996). She found that YA-deficient eggs and embryos appear to complete meiosis. Since completion of meiosis by Drosophila eggs requires activation, but not fertilization, she performed FISH comparisons of control and Ya^2 laid (activated) eggs. Each of the various categories of associated nuclei observed in Ya^2 eggs requires, or is consistent with, normal completion of meiosis. Sixty-eight percent of Ya^2 eggs contain at least one haploid nucleus based on FISH, thus demonstrating completion of meiosis. The conformations of nuclei in YA-deficient eggs favor the model that Ya^2 eggs complete meiosis and then undergo nuclear associations, and the data from YA-deficient embryos also support this model. In this chapter I show that in addition to completion of meiosis, other events of egg activation occur in YA-deficient embryos. Crosslinking of the vitelline membrane occurs in Ya^2 embryos, as does egg-activation dependent protein translation, and GNU and ERK dephosphorylation.

Prior characterizations of Ya^2 eggs and embryos made assumptions about pronuclear association based on relative DNA intensities or identifiable structures such as the polar body rosette and apposed pronuclei positioned in the anterior center of the embryo. Using FISH to distinguish the paternal pronucleus, J. Lopez showed that when Ya^2 eggs are fertilized, the resulting embryo can either resemble a wildtype embryo prior to the gonomeric mitosis, or its pronuclei can associate abnormally. In over half the Ya^2 embryos, the male pronucleus does not associate with a female pronucleus. In some cases the male pronucleus associates with pairs of maternally-derived haploid nuclei. These data suggest that in the absence of YA function, the maternally- and paternally-derived haploid nuclei lose identity (eg. as a polar body vs.

a pronucleus). The maternal haploid meiotic products establish identity as they become either the female pronucleus or the polar bodies, and the male pronucleus has a distinct identity although its chromatin proteins and nuclear envelope are maternally provided. YA's localization to the nuclear lamina (Lopez et al. 1997) may suggest a role in pronuclear identity or pronuclear apposition. Lamina characteristics specific to a given nuclear identity could function in appropriate nuclear associations.

Although the nuclear association defects in Ya^2 are similar in timing to the association defects in mutants of three genes, *KLP3A, asp,* and *ncd* that encode microtubule-related proteins, and to mutants of the kinase *polo* that also have microtublule-based nuclear association defects. YA's nuclear localization suggests a different basis for the arrest. Because pronuclear migration is microtubule-dependent, the association defects of *KLP3A, asp, ncd,* and *polo* mutants are considered to result from defects in nuclear migration. The male pronucleus does not associate properly with a single female pronucleus in *Ya, KLP3A,* or *asp* mutant embryos, although in *KLP3A* embryos the four female meiotic products always form a single rosette instead of the various combinations seen in Ya^2 embryos, and in *asp* embryos the chromatin of the polar bodies also condenses normally (Riparbelli et al. 2002; Williams et al. 1997). In *ncd* and *polo* mutant embryos the male pronucleus remains unassociated, and all the female polar bodies undergo repeated haploid mitosis (Endow and Komma 1998; Riparbelli et al. 2000). In this chapter I show that the sperm aster can form in YA-deficient embryos, permitting microtubule-based nuclear migration in these mutant embryos.

A unique cell cycle transition occurs following the resumption of meiosis upon egg activation: from completion of meiosis to the first mitotic division. Severe *Ya* mutants fail to initiate the first embryonic mitosis and were reported to arrest with apposed pronuclei (Lin and Wolfner 1991; Liu et al. 1995). Since pronuclear

apposition in embryos usually coincides with the post-meiotic S phase, but the apposed pronuclei in *Ya* mutant embryos have condensed chromatin and so do not appear to be in S phase, *Ya* mutants may be arrested prior to or after the first S phase. (Arrest during S phase with abnormal condensation is also a possibility.) *Ya* null mutations are epistatic to *plu, gnu* and *png* mutations; double mutants arrest with the chromatin condensation phenotype of the *Ya* mutant instead of forming the giant nuclei characteristic of the *plu/gnu/png* complex mutants (Liu et al. 1997; Shamanski et al. 1991). This epistasis suggests that YA could be required to initiate the repeated cell cycles, but the time in the cell cycle when YA functions remained unclear. The data in this chapter suggest that S phase occurs, or at least begins, in the absence of YA function, since the replication fork component PCNA, and the canonical Histone H3, which is deposited in a replication-dependent manner, are both detected on chromatin of some nuclei in YA-deficient eggs and embryos. However, the cell-cycle stage of egg and embryo nuclei, assessed with phospho-histone H3 and PCNA, and the cell-cycle dependent chromatin condensation state, are asynchronous in the absence of YA function. The asynchrony and the abnormal nuclear association phenotypes, together with the arrest prior to the first mitosis, suggest that YA functions in the nucleus to facilitate the nuclear identities and synchrony of the male and female pronuclei, and acts upstream of a cell-cycle checkpoint that is activated in response to loss of nuclear identity and/or synchrony.

5.2 Materials and Methods

Drosophila strains:

The apparent null allele Ya^2 (H. F. Lin et al. 1991) was carried in stock as ***X^X,*** y^2 *fs(1)*Ya^2 w^{bf}*spl* sn^3/*Y,*y^+ *fs(1)*$Ya^+$$w^+$*B* (abbreviated as ***X^X,*** Ya^2 and *Y,* Ya^+, respectively). YA-deficient eggs or embryos (referred to here as "Ya^2 eggs" or "Ya^2 embryos", respectively) were generated from ***X^X,*** Ya^2 females carrying a normal Y

(daughters of females from the stock crossed to Oregon R P2 males). Oregon R P2 embryos were the control (Allis et al. 1977). The histone H3-FLAG and HIRA-FLAG transgenic stocks were a gift from B. Loppin (Loppin, Bonnefoy, et al. 2005), and YA-deficient embryos expressing the H3-FLAG or HIRA-FLAG transgene from one allele were generated by crossing males from one of these stocks to females of the *Ya*^{2} stock as described above. For collections of unfertilized eggs for immunofluorescence, *X^X,* *Ya*^{2} females carrying a normal Y were mated to spermless sons of *tudor*^{1} *bw sp* females (Boswell et al. 1985). Flies were raised at room temperature (≈23˚C) with a 12:12 hour light: dark cycle on yeast-glucose media.

Protein extraction and western blots:

Dissected mature oocyte (stage 14) or laid embryos samples were homogenized in protease inhibiting homogenization buffer [PIHB; (Monsma et al. 1988)] with the addition of two phosphatase inhibitors, 20mM sodium fluoride and 10mM β-glycerophosphate. Total protein was quantified by Bio-Rad Protein Assay (#500-0006, Bio-Rad Laboratories, CA). Samples were prepared for gel electrophoresis by boiling in sample-buffer, and 20-60 micrograms of protein were loaded into each lane of a 10.6% SDS polyacrylamide gel. Western blots were prepared as previously described. Anti- (total) ERK, anti-phospho-ERK, (Cell Signaling Technology, MA) were both used at a dilution of 1:1000, according to manufacturer's recommendations. We used anti- (total) ERK staining as a loading control. Anti-α-tubulin antibody (Sigma, catalog #T5168) was diluted 1:10,000. Guinea-pig anti-GNU [gift of T. Orr-Weaver, (Lee et al. 2003)] was diluted 1:5000. Guinea-pig anti-SMG [gift of W. Tadros and H. Lipshitz (Tadros et al. 2007)] was diluted 1:10,000. After incubation with primary antibody overnight at 4°C, blots were probed with anti-rabbit, -guinea pig, or -mouse HRP secondary antibodies (Jackson Immunochemicals, PA) at a 1:2000 dilution, according to manufacturer's

recommendation. Signals were detected with ECL or ECL-Plus (GE Healthcare, United Kingdom).

Immunofluorescence:

X^X, *Ya*^{2}/Y or Oregon R P2 females were mated to Oregon R P2 males for 0-15 minute collections of *Ya*^{2} mutant or control embryos. Laid unfertilized eggs were collected from unmated females or females mated to spermless males (sons of wildtype males x *tud*^{1} *bw sp* females) (Boswell et al. 1985). Embryos fixed in methanol/heptane were rehydrated in PBST. Anti-phospho histone H3 (Upstate) or anti-PCNA [gift of P. Fisher, SUNY StonyBrook (Ng et al. 1990)] were used at 1:100 or 1:50 dilutions, respectively, in PBST. Anti-α-tubulin antibody (Sigma, catalog #T5168) was diluted 1:100, Anti-acetyl H4 (K5,8, 12,16) was diluted 1:600. Eggs or embryos laid by H3-FLAG or Hira-FLAG control females or X^X, *Ya*^{2}/Y ; H3-FLAG or X^X, *Ya*^{2}/Y ; Hira-FLAG mutant females were fixed as above and anti-FLAG (Sigma) was used at a 1:400 dilution. Secondary was Alexa 488- or Alexa 633-conjugated anti-rabbit or anti-mouse (Invitrogen, Carlsbad, CA). DNA was stained with 10μg/ml propidium iodide or a 1:750 dilution of Oligreen (Invitrogen) stock solution. Fixed and stained samples were mounted in (75% glycerol containing 940mM *n*-propyl gallate) or washed in MeOH and mounted in 2:1 benzyl benzoate: benzyl alcohol (Theurkauf 1994). Eggs and embryos were analyzed using confocal microscopy (Leica TCS SP2 system equipped with an argon-krypton laser and coupled to a Leica DMRBE microscope). Leica software was used to collect images. Where appropriate, Leica software was used to project multiple optical sections into a single image and to overlay images.

5.3 Results

Four aspects of egg activation occur normally in YA-deficient embryos:

Meiosis can complete in the absence of YA function (J. Lopez, dissertation, and C. Berman, thesis), so I assayed whether other aspects of egg activation can occur. When vitelline membrane protein cross-linking occurs, the Drosophila eggshell becomes impermeable to small molecules, including bleach, but 50% bleach lyses most unactivated eggs/embryos within two minutes (Heifetz et al. 2001; Mahowald et al. 1983). The percentage of embryos that resist lysis in bleach is not significantly different between embryos laid by *Ya²* mutant females (X^X *Ya²*/Y) and those laid by control females from the stock (X^X *Ya²*/*Ya*⁺Y) who have a 50%-shared strain background (Fig 5.1A). The SMAUG protein is translated upon activation (Tadros et al. 2007) in *Ya²* mutant embryos as in controls (Fig 5.1B). Levels of phospho-ERK decrease upon egg activation in *Ya²* mutant embryos as in controls, while total ERK levels remain the same before and after egg activation (Fig 5.1C). GNU is also dephosphorylated upon egg activation (Renault et al. 2003) in *Ya²* mutant embryos as in controls, such that its electrophoretic mobility increases, so it migrates lower on the gel (Fig 5.1D).

Three characteristics of the male pronucleus are normal in *Ya²* embryos:

The male pronucleus can be apposed to and associate with one (or more) female meiotic products in *Ya* embryos, and the female pronucleus does not undergo independent haploid mitotic divisions that often occur in mutants that affect the male pronuclear function. To further confirm that *Ya* function is first required for the first mitosis for the paternal pronucleus as for the maternal pronucleus, I tested for three earlier aspects of male pronuclear reorganization in *Ya* embryos. Microtubule spindles of meiotic, mitotic, and abnormal configurations have been seen in YA-deficient embryos [(Lin et al. 1991), Cindy Berman Thesis], but the sperm aster had not yet

A

Female genotype	Eggshell hardened	Number of eggs	Percent
control	131	131	100%
Ya^2	123	124	99%

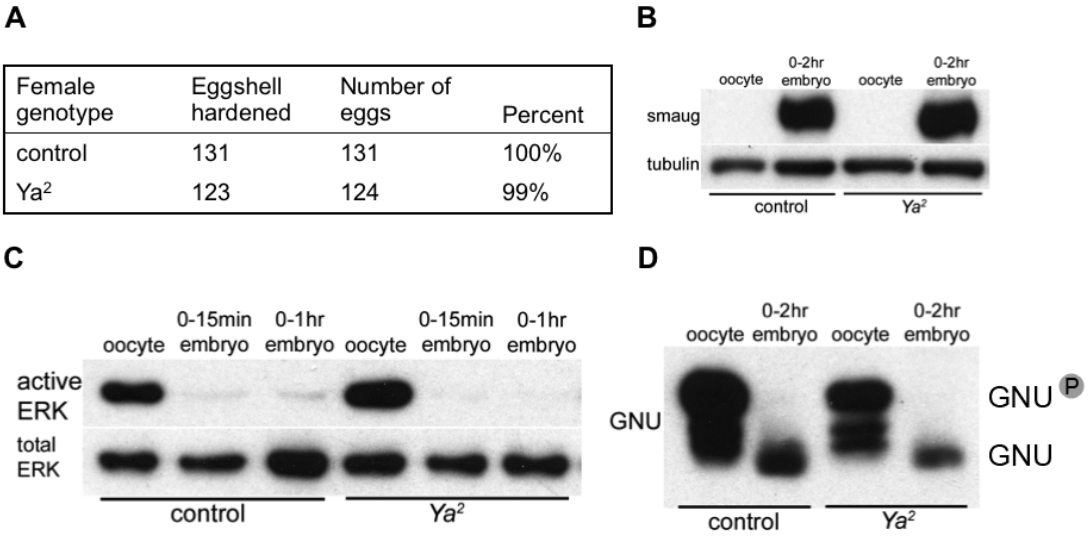


Figure 5.1: **Four aspects of egg activation are normal in YA-deficient embryos.** (A) The percentage of laid embryos impermeable to 50% bleach after a 2-minute incubation was not significantly different between control and *Ya* mutant mothers. (B) Translation of the SMAUG (SMG) protein after egg activation was assayed by western blotting with anti-SMG. The loading control is anti-α-tubulin. SMG levels in 0-2hr-old embryos are shown for control and *Ya* mutant. (C) Dephosphorylation of the MAP kinase ERK upon egg activation was assayed by western blotting with anti-phospho-ERK antibody. Anti-total ERK was used as a loading control. Phospho-ERK levels decrease upon egg activation in *Ya* mutant embryos as in controls. (D) Electrophoretic mobility of GNU upon egg activation was assayed by western blotting with anti-GNU. Phosphorylated GNU in oocytes has a slower electrophoretic mobility than dephosphorylated GNU in embryos (Renault et al. 2003), and the dephosphorylated form is present in *Ya* mutant embryos as in control embryos.

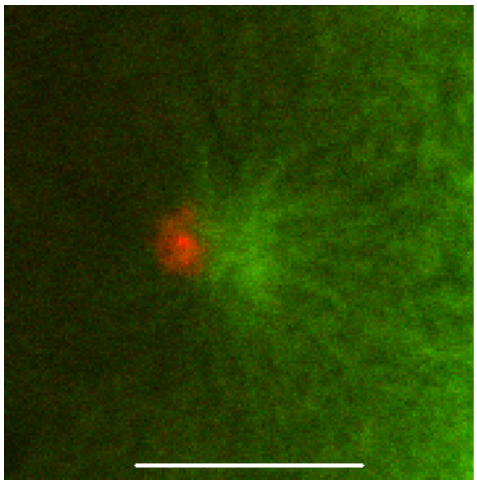
been examined. The presence of normal female microtubule structures does not imply that the male microtubule structures are also normal. For example, the formation of the microtubule aster nucleated by the sperm nucleus does not occur normally in embryos deficient for the egg activation gene *sarah*, although the meiotic spindle(s) are observed (Horner et al. 2006). Using anti-alpha-tubulin I observed a normal sperm aster in YA-deficient embryos (3 of 3 identifiable male nuclei observed) (Fig 5.2A). Therefore YA function is not required for the male nucleus to organize its microtubule aster that is necessary for pronuclear migration.

The reorganization of the sperm nucleus into the male pronucleus includes changes in chromatin state. Prior to the first mitosis in Drosophila, only the male pronucleus stains for acetylated histone H4, and the histone H3.3 chaperone HIRA (Loppin, Bonnefoy, et al. 2005) localizes only to the male pronucleus in wildtype embryos. I observed localization of both these markers to only one nucleus in *Ya* embryos (Fig 5.2B,C). The one nucleus with acetylated histone H4 or HIRA is presumably the male nucleus, and in most embryos the characteristic needle- or small rounded- shape of the sperm nucleus before or early in chromatin remodeling provides further evidence that the observed staining is specific to the male nucleus. The positions of nuclei in the embryo may also suggest nuclear identity based on characteristic positioning of female meiotic products and the male pronucleus. However, in embryos where fewer than five nuclei are observed (suggesting nuclear associations have occurred) and the nuclei are all condensed, the identification of the one nucleus with HIRA as the male nucleus is not definite. The simplest explanation for the observation of only one acetyl-H4 staining or HIRA-staining nucleus per *Ya* embryo is that YA function is not required for male-specific chromatin remodeling to occur.

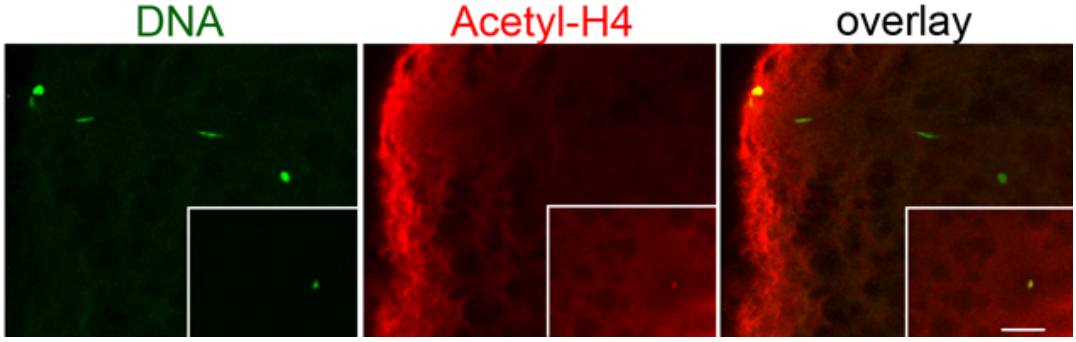
Figure 5.2: The male nucleus in YA-deficient embryos

Immunostaining of 0-15 minute collections of laid *Ya²* embryos (from X^X *Ya²*/Y or X^X *Ya²*/Y ; HIRA-FLAG mothers) includes embryos just completing female meiosis (A&B), and during pronuclear migration (C). (A) Sperm Aster: Microtubules shown in green, DNA in red. *Ya²* n=3 asters observed out of 3 sperm nuclei identified. (B) Acetyl-histone H4: DNA in green, Acetyl-H4 in red. The one nucleus that stains for acetyl-H4 was in a quite different focal plane from the set of acetyl-H4 negative nuclei and is therefore shown in an inset, and is probably the male pronucleus. *Ya²* n=7 (C) *Ya²* embryos laid by X^X *Ya²*/Y ;HIRA-FLAG females: DNA shown in green, anti-FLAG shown in red. The one nucleus that stains for HIRA-FLAG (male pronucleus) was in closer proximity to a non-HIRA-FLAG staining nucleus (probably the female pronucleus), than to two other non-staining (polar body) nuclei shown in the inset. *Ya²* n=1. Bar = 10 μm.

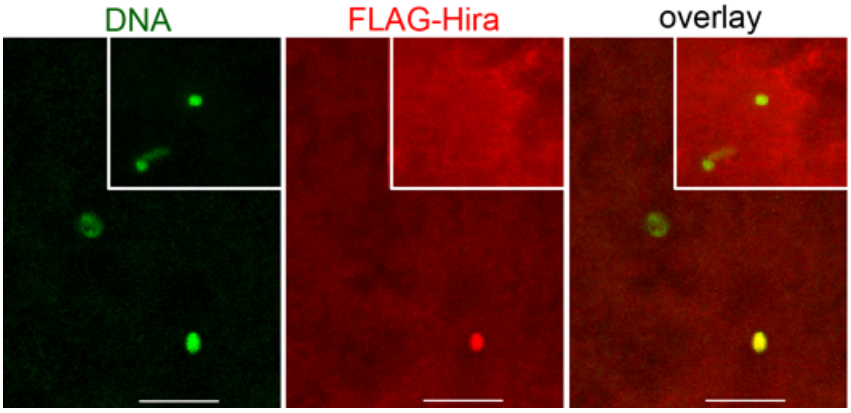
A



B



C



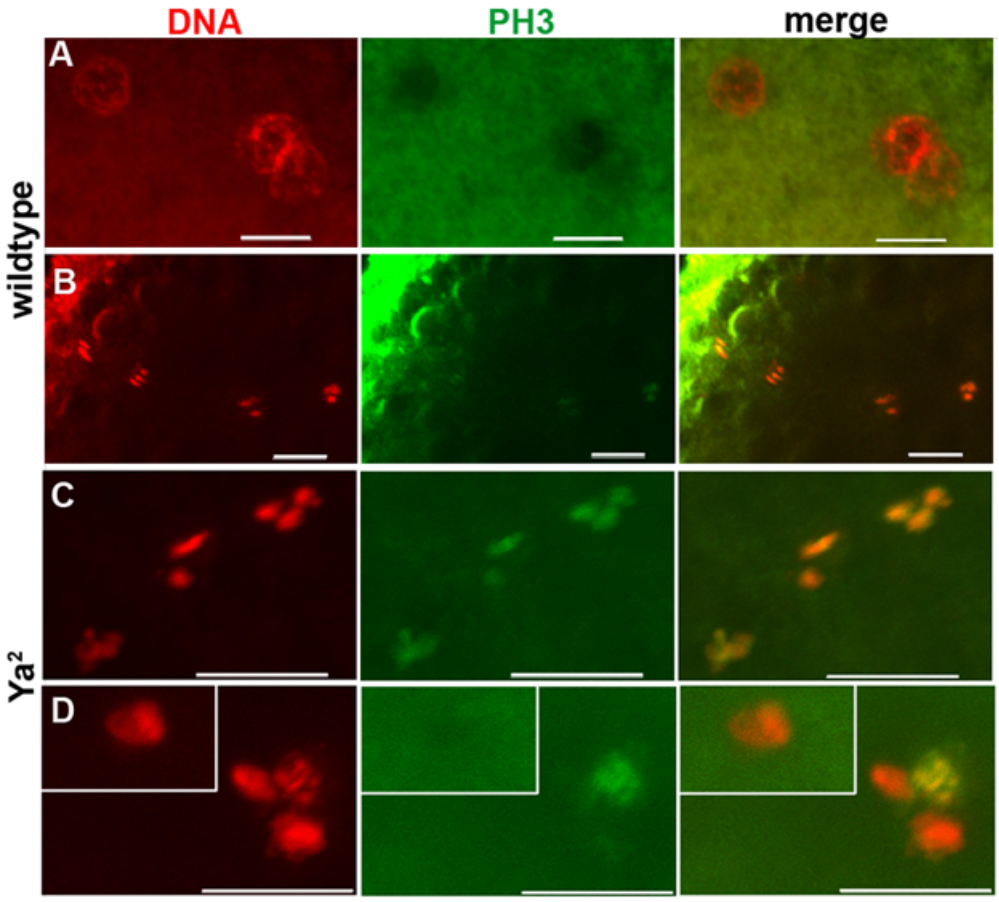
Meiotic products in wildtype eggs and embryos are synchronous in PCNA and PH3 distribution:

The earliest phenotypes of *Ya²* embryos are the non-wildtype nuclear associations. A previous report using DAPI staining (Liu et al. 1995) suggested that nuclear condensation states were asynchronous in *Ya²* embryos but could not distinguish the compositions of the highly-condensed *Ya²* mutant nuclei or make any conclusions about cell cycle state. To determine more precisely the nature and consequences of this asynchrony, I characterized the chromatin state of haploid meiotic products prior to the gonomeric division using antibodies to cell-cycle specific features. I tested for phospho-serine 10 of histone H3 (PH3), a histone modification characteristic of mitotic chromatin (Nowak et al. 2004), and for Proliferating Cell Nuclear Antigen (PCNA), a component of the replication complex and therefore a marker of S phase (Yamaguchi et al. 1991).

Since wildtype nuclei have defined identities and cell cycle states in the transition from meiosis to mitosis, I began by characterizing the localization of phospho-histone H3 and PCNA at this developmental stage. In syncytial embryos, PH3 staining colocalizes exactly with mitotic nuclei (Su et al. 1998), while PCNA colocalizes with replicating nuclei, and may persist on chromatin into prophase; it is known to be undetectable by metaphase (Yamaguchi et al. 1991). The condensed chromatin of the polar bodies and mitotic gonomeric nucleus in wildtype eggs (Fig 5.3 A, B) and embryos (Fig 5.4A shows apposed pronuclei) stains with PH3 but not PCNA, as expected. In contrast, when all the haploid or mitotic nuclei were decondensed, no PH3 staining was seen on the chromatin (any faint PH3 staining was around the nuclear periphery). Decondensed nuclei showed two main types of chromatin patterns, along with some rare apparent intermediates: either a sphere with soft, undefined edges and an average 5.7 μm diameter, or a ring of stringy chromatin

Figure 5.3: Phospho-histone H3 distribution on chromatin of unfertilized wildtype and YA-deficient eggs

Immunostaining of 0-15 minute old laid unfertilized wildtype (Oregon R P2) and *Ya²* (X^X *Ya²*/Y) eggs. DNA shown in red (propidium iodide staining), phospho-histone H3 (PH3) shown in green. A-I are projections of multiple confocal images. (A) Four decondensed wildtype nuclei in promeiotic interphase or S phase with PH3 excluded from nuclei. (B) Four condensed wildtype meiotic products perpendicular to the egg cortex with PH3 staining on all chromatin. (C) Condensed *Ya²* meiotic products with PH3 staining on all chromatin. (D) Four *Ya²* meiotic products in two planes; three grouped at the egg cortex and one deeper in (inset). Only one of the four has individualized chromosomes with PH3 staining, although all four nuclei have similar nuclear areas, and thus are likely all haploid. Wildtype n=64 eggs. *Ya²* n=20 eggs. Bar = 10 μm.


DNA
PH3
merge
A
B
C
D
wildtype
Ya2

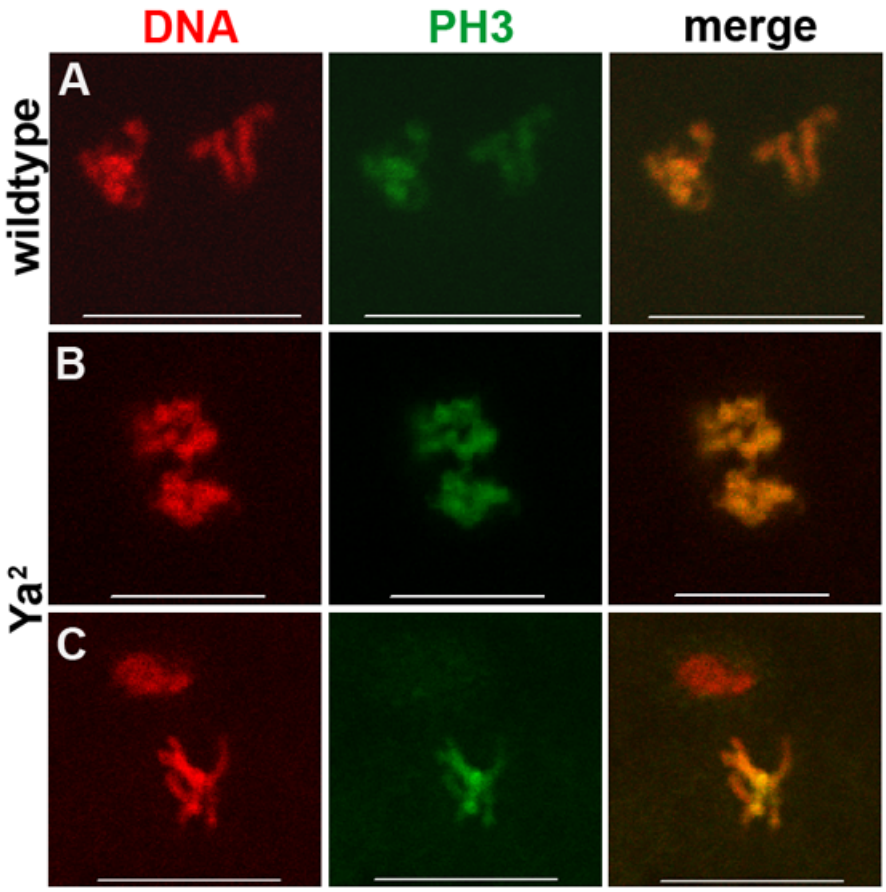


Figure 5.4: Phospho-histone H3 distribution on chromatin of wildtype and YA-deficient embryos

Immunostaining of 0-15 minute old wildtype (ORP2) and *Ya²* embryos. DNA shown in red (propidium iodide staining), phospho-histone H3 shown in green. Embryos shown had three peripheral nuclei (not shown) and two nuclei apposed in the mid-anterior region. Apposed nuclei are shown. (A) Wildtype, (B) Synchronous apposed nuclei in a *Ya²* embryo (70%), (C) Asynchronous apposed nuclei in a *Ya²* embryo (30%). Wildtype n=22 embryos with apposed pronuclei. *Ya²* n=27 embryos with apposed nuclei. Bar = 10 μm.

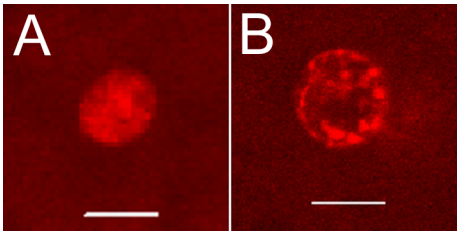


Figure 5.5: **Variation in decondensed nuclear morphology.**

DNA (propidium iodide staining) of wildtype 0-15 minute old embryos shown in red. Neither of these example nuclei had PCNA localized to their chromatin. (A) Soft, round nucleus, n=15. (B) Stringy ring nucleus, n=11. Bar = 6 μm

primarily along the nuclear periphery with an average 7.5 μm diameter (Fig 5.5). A significant number of "soft sphere" nuclei and the majority of "stringy ring" nuclei had PCNA colocalized with the chromatin (Fig 5.6 A, C). While it is possible that this varied nuclear morphology is a fixation artifact (Foe et al. 1985), I interpret the two types of PCNA-staining nuclei as S phase or early prophase chromatin, and the two types of PCNA-negative nuclei as late prophase or G2-like.

Meiotic products in some YA-deficient eggs and embryos are asynchronous in PCNA and PH3 distribution:

The haploid nuclei in *Ya²* eggs and embryos can associate in both normal and abnormal combinations as described above. In addition, when observed by DAPI or propidium iodide staining these nuclei have a variety of chromatin configurations, primarily overcondensed (Lin et al. 1991). The appearance of individual nuclei varies within a single mutant embryo. Some differences in size are attributable to differences in ploidy, but condensation states of nuclei can also vary within the same embryo (Lin et al. 1991; Liu et al. 1995). Localization of phospho-histone H3 and PCNA staining in these YA-deficient eggs and embryos highlights the asynchrony between the various nuclei and demonstrates that the asynchronous chromatin condensation may reflect asynchronous cell-cycle states.

Phospho-histone H3 (PH3) is detected in the condensed and overcondensed nuclei of *Ya²* eggs and embryos, indicating that their chromatin is in a mitotic-like state (Figs 5.3, 5.4). The chromatin of polar body rosettes in both mutant and wildtype also stains for PH3, although polar bodies are not actively mitotic. When the nuclei are decondensed, PH3 is not visible on the chromatin. Cell-cycle asynchrony of nuclear chromatin in a given egg or embryo is observed: in 25% of *Ya²* eggs and 6% of *Ya²* embryos, some nuclei are condensed and have PH3 staining, while one or more other nuclei are not (total *Ya²* embryos observed n=136). Though eggs and embryos were

collected within 15 minutes of oviposition, the majority of nuclei are condensed, displaying the "pronuclear arrest" *Ya²* phenotype (Liu et al. 1995), and in 94% of the mutant embryos all the condensed nuclei stain for PH3. In the rare cases where three haploid nuclei migrate to the periphery and two are apposed at the center of the *Ya²* embryo (n=27 out of 136 *Ya²* embryos), we have also observed asynchrony between the two apposed nuclei, with only one of the two staining for PH3 (Fig 5.4 C, 30% asynchrony of apposed nuclei, 8 out of 27). We were not able to distinguish male- from female-derived pronuclei in these experiments. Asynchrony characterized by PH3 localization to some nuclei but not others is never seen in wildtype eggs or embryos prior to or during the gonomeric division; the polar bodies and pronuclei are coordinated. However, in wildtype embryos during the syncytial divisions the polar body chromatin is always mitotic-like, as mentioned above, and so stains for PH3 even when the dividing nuclei are decondensed and do not have phosphorylated histone H3: we did not consider this to be asynchrony when observed in wildtype or *Ya²* embryos.

PCNA localization in *Ya²* embryos also demonstrates asynchrony (Fig 5.6). The chromatin of condensed *Ya²* embryo nuclei does not usually have visible PCNA staining, whereas some of the *Ya²* embryo decondensed nuclei have PCNA colocalized with their chromatin. The decondensed PCNA-positive *Ya²* nuclei can resemble either "soft sphere" or "stringy ring" wildtype decondensed nuclei, but may not actually be progressing through S phase. In most *Ya²* embryos all the nuclei are condensed and do not stain for PCNA, but in some cases all the nuclei are decondensed and contain PCNA. In these fixed collections of embryos there are more embryos with condensed nuclei than decondensed nuclei because the final *Ya* arrest phenotype is condensed chromatin, and the decondensed state is probably transient. In contrast to those *Ya²* embryos where the chromatin condensation is synchronous (resembling wildtype), the *Ya²* embryos with asynchronous nuclei show asynchronous PCNA localization, neither

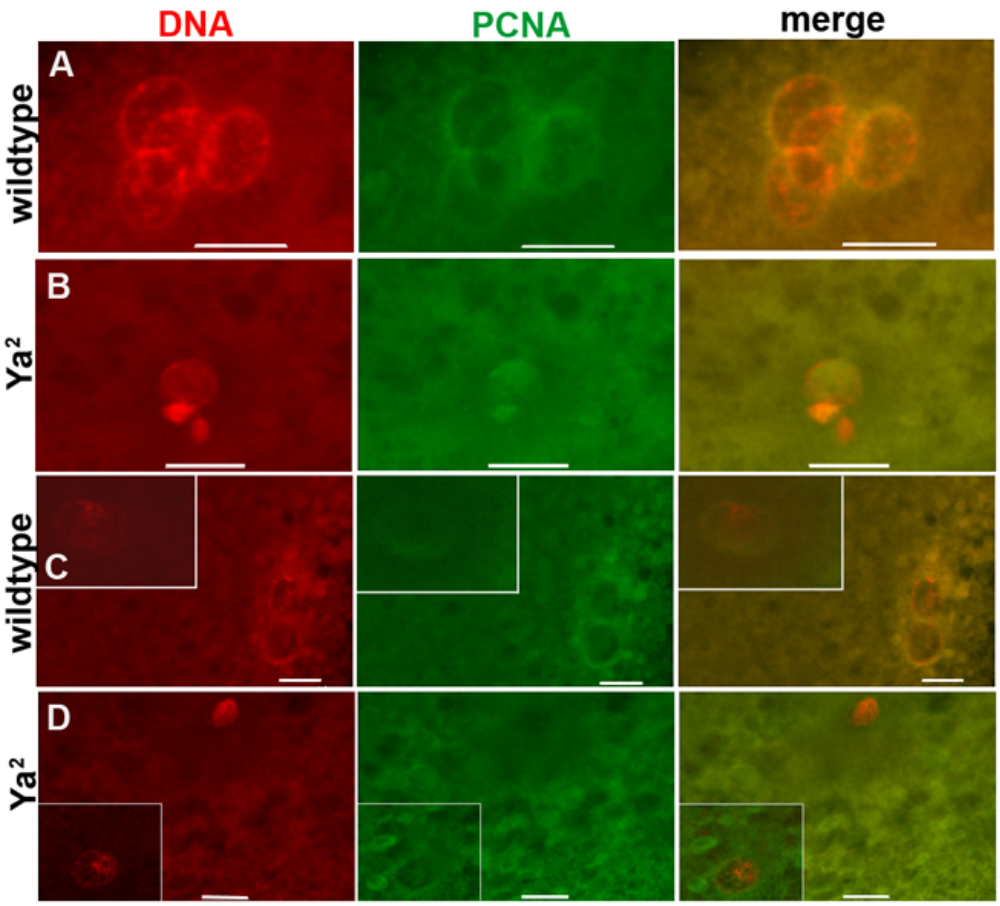
of which are seen in wildtype embryos. To ensure all nuclei present were detected by immunofluorescence, we only tallied embryos with five meiotic products, thus before any nuclear associations. In 41% of these *Ya²* embryos some nuclei have PCNA localized to chromatin while other nuclei do not, although the numbers of nuclei out of the five that are PCNA-staining and not PCNA-staining vary between embryos. These immunofluorescence data highlight the nuclear asynchrony in YA-deficient embryos. Within a single *Ya²* egg or embryo, nuclei with different degrees of chromatin condensation may also be at different phases of the cell cycle, although cell-cycle asynchrony never occurs between nuclei within a wildtype egg or embryo at this point in development.

Replication occurs in YA-deficient eggs and embryos:

The presence of PCNA on the chromatin of YA-deficient eggs and embryos suggests they may at least begin DNA replication. In order to assay for DNA replication, BrdU is commonly introduced into cells where it is incorporated into replicating DNA and can be detected by anti-BrdU antibody. Because BrdU incorporation is undetectable in individual meiotic products (Appendix D), to independently assay for replication I performed immunofluorescence using transgenic flies bearing a FLAG-tagged version of canonical histone H3 (Loppin, Bonnefoy, et al. 2005), which is deposited in a replication-dependent process. Anti-FLAG immunofluorescence does not detect other H3 variants that would also react with available histone H3 antibodies. Since some other forms of histone H3, such as H3.3, are deposited in a replication-independent manner (Konev et al. 2007), if they were detected by anti-H3 antibody along with canonical H3, one could not draw conclusions about replication. Therefore, I used anti-FLAG to detect transgenic H3-FLAG, and observation of FLAG-H3 incorporation into DNA serves as a proxy for directly measuring DNA replication. In wildtype embryos, maternally-derived histone

Figure 5.6: PCNA distribution on chromatin of wildtype and YA-deficient eggs and embryos

Immunostaining of 0-15 minute old laid unfertilized wildtype and *Ya*^{2} eggs, and wildtype and *Ya*^{2} embryos. DNA shown in red, PCNA shown in green. A-F are projections of multiple confocal images. (A) Egg: Four decondensed wildtype nuclei in promeiotic interphase or S phase with PCNA on DNA. (B) Egg: variously condensed *Ya*^{2} meiotic products with PCNA staining on 2 out of 3 areas of chromatin. (C,D embryos) Due to the very faint immunofluorescence of some nuclei, embryos were only considered synchronous when they had five visible nuclei. However, asynchrony can be evident with as few as two nuclei. (C) Embryo: Five decondensed wildtype meiotic products with PCNA staining on all nuclei. Two apposed pronuclei (inset) and three meiotic products forming a polar body (only two are visible in this plane). (D) Two *Ya*^{2} nuclei in two planes; one near the embryo cortex (inset) and one deeper in. Only one of the two has PCNA staining. Wildtype n=40 eggs. *Ya*^{2} n=31 eggs. Wildtype n=102 embryos. *Ya*^{2} n=41 embryos. Bar = 10 μm.


DNA
PCNA
merge
A
wildtype
B
Ya²
C
wildtype
D
Ya²

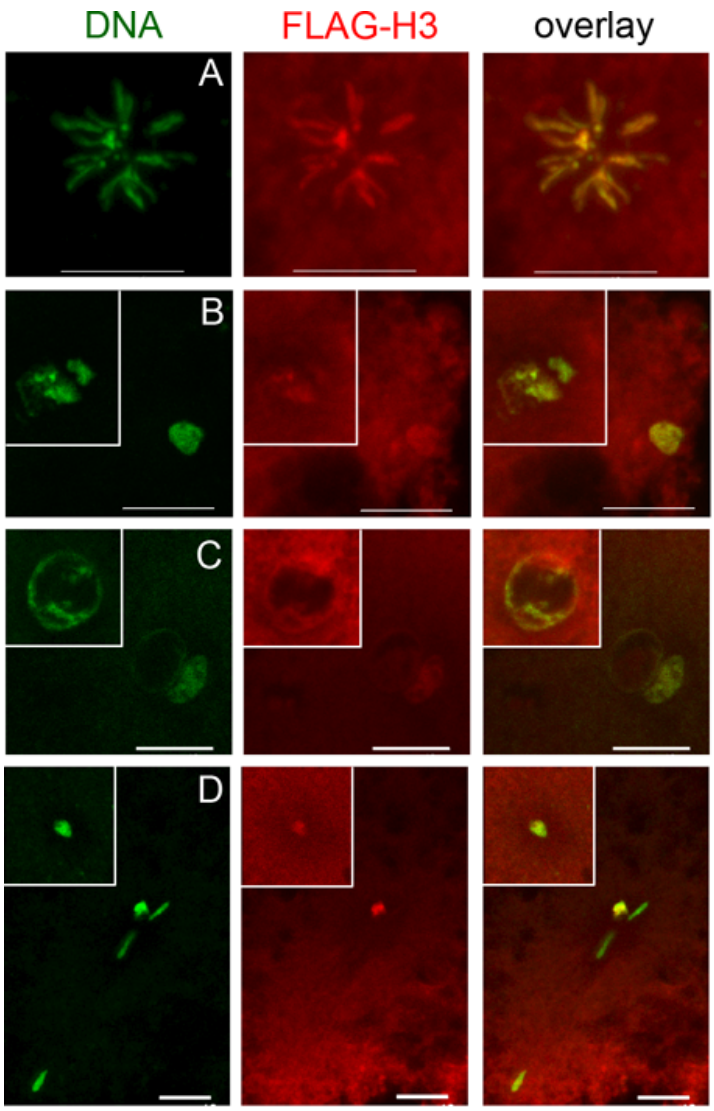
H3-FLAG is first present on the sperm nucleus and is first visible on female meiotic products after the first post-meiotic S phase (Loppin, Bonnefoy, et al. 2005). Histone H3-FLAG is detectable on some fraction of nuclei (Figure 5.7) in the majority of YA-deficient eggs (54%) and embryos (93%), suggesting that at least some replication has occurred in order for histone H3-FLAG to become detectable on female-derived nuclei, and for H3-FLAG to have been incorporated into male-derived nuclei. The histone H3-FLAG can either be present on all the chromatin of a YA-deficient egg (A, B) or embryo (C), or it can be on some nuclei but not others, suggesting asynchronous replication (10/56 *Ya* embryos, 1/20 *Ya* eggs) (D). If the inset nucleus in (D) is the male nucleus, as its position in the embryo suggests, then there is asynchrony between the male nucleus and three of the female nuclei, but also asynchrony between the female nuclei in this YA-deficient embryo.

5.4 Discussion

Ya mutant embryos undergo the events of egg activation normally. In addition to vitelline membrane protein cross-linking, SMAUG translation, and protein dephosphorylation (all reported here), meiosis completes (J. Lopez, Dissertation, and C. Berman, Thesis) and maternal transcripts are dephosphorylated (Tadros et al. 2003). I have shown that the sperm aster can form in YA-deficient embryos, and that both acetyl-histone H4 and HIRA are present on the male pronucleus in YA-deficient embryos, indicating proper remodeling of the sperm nucleus into the male pronucleus. In the absence of YA function, the chromatin of egg and embryo nuclei can stain with PCNA and be somewhat decondensed before arriving at the ultracondensed chromatin mutant phenotype. In 0-15 minute collections of Ya^2 mutant embryos, individual nuclei can be at one of a few different cell cycle stages. These nuclei can be either condensed and mitotic-like as determined by the presence of phospho-histone H3, or they can be G2-like decondensed nuclei, or decondensed S-phase-like nuclei as

Figure 5.7: Histone H3-FLAG distribution on chromatin of YA-deficient eggs and embryos.

Immunostaining of 0-15 minute old laid unfertilized H3-FLAG; *Ya*^{2} eggs, and H3-FLAG;*Ya*^{2} embryos. DNA shown in red, H3-FLAG shown in green. A-D are projections of multiple confocal images, with insets used to show nuclei in a greatly different focal plane. (A) Egg with polar body rosette with H3-FLAG staining. (B) Egg with four female meiotic products all with H3-FLAG staining. (C) Embryo with two apposed nuclei and a third nucleus nearer the egg cortex, all with H3-FLAG staining. (D) Embryo with five nuclei, two of which are more rounded and have H3-FLAG staining while three others do not. The H3-FLAG-staining nucleus not in the inset is closer to the three unstained nuclei along the Z axis than it is to the other H3-FLAG-staining nucleus shown in the inset. *Ya*^{2} eggs n=24. *Ya*^{2} embryos n=58. Bar = 10 μm.


DNA
FLAG-H3
overlay
A
B
C
D

indicated by the presence of PCNA. In longer (0-2 hr) collections, most of the chromatin in *Ya²* embryos looks abnormally condensed and more nuclear associations have occurred (Liu et al. 1995). At least some DNA replication occurs, since the presence of PCNA is permissive of replication, and incorporation of histone H3-FLAG is indicative of at least some replication having occurred. *Ya²* embryos are probably limited to at most one S phase prior to the gonomeric division, because *Ya²* is epistatic to *gnu* and *plu* (Liu et al. 1997; Shamanski et al. 1991). Based on the PCNA and H3-FLAG staining, YA function may not be required to start the pre-gonomeric S phase, but it could promote or maintain appropriate nuclear associations and chromatin condensation state which would allow progression of the first mitosis or allow S phase to complete.

In *Ya²* eggs and embryos with abnormal nuclear associations, the two, three or four nuclei do not necessarily share chromatin condensation state or cell-cycle stage based on phospho-histone H3 or PCNA staining. Even *Ya²* eggs with four meiotic products or *Ya²* embryos with five haploid nuclei, which have avoided abnormal associations, can have asynchronous chromatin condensation state of their nuclei, in contrast to wildtype. PCNA staining in *Ya²* eggs or embryos is usually on their nuclei with decondensed chromatin and phospho-histone H3 is usually only on their condensed nuclei. So nuclei within the same *Ya²* egg or embryo can differ in PH3 or PCNA staining. Since such asynchrony is seen in *Ya²* unfertilized eggs, it cannot simply be a distinction between maternally- and paternally-derived nuclei, although we cannot distinguish maternal from paternal chromatin in most *Ya²* embryos. Of the different classes of *Ya²* eggs and embryos distinguished by FISH, only in those with either all the nuclei associated, all the nuclei remaining distinct, or three nuclei at the periphery and two associated pronuclei within the embryo can nuclear identities be determined with some confidence by DNA staining and immunofluorescence, since

estimating nuclear ploidy by size and DNA intensity is confounded by the asynchronous condensation states of the nuclei. Therefore I cannot correlate asynchronous cell cycle state and abnormal nuclear associations. The *Ya*^{2} arrest at the transition from meiosis to mitosis suggests activation of a checkpoint that could prevent zygotes with five uncoordinated or improperly associated haploid nuclei from proceeding through development. When this proposed checkpoint is triggered in the absence of *Ya* function, S phase can still occur, suggesting that the checkpoint arrests the cell cycle by regulating S-->(G2)--> M phase molecules rather than molecules involved in the meiosis-->(G1)--->S phase transition.

The loss of nuclear identity as seen by abnormal associations of haploid nuclei is a phenotype that suggests disruption of molecules associated with the nuclear envelope, because the nuclear envelope is the outer layer of the nucleus that contains molecules that could interact with molecules on other nuclear envelopes. The timing of the requirement for YA after activation is coincident with its localization to the nucleus and nuclear lamina upon completion of meiosis when the meiotic products decondense their chromatin (Liu et al. 1997; Lopez et al. 1994). YA's ability to interact with chromatin (Lopez et al. 1997; Yu and Wolfner 2002) is ideally suited to sense/regulate/transduce condensation state. The nuclear lamina is involved in regulating S phase (reviewed in Moir et al. 2000), and YA is in both the nuclear lamina and the nucleoplasm. YA may function through its binding to lamin (Lopez et al. 1994), DNA, and histone H2B (Yu and Wolfner 2002) to detect and maintain a coordinated chromatin state of the male and female pronuclei appropriate for the gonomeric mitosis. I do not know how the chromatin asynchrony observed in *Ya*^{2} embryos relates to the improper nuclear associations seen there, but both are characteristics of the absence of YA function. In the presence of asynchronous meiotic products or apposed pronuclei, our proposed gonomeric mitosis checkpoint would be

active. YA's interaction with chromatin may be appropriate for creating, maintaining or restoring chromatin synchrony to permit the gonomeric mitosis. Some differences between pronuclei are normal, such as distinct chromatin methylations or acetylations observed in mice (Sarmento et al. 2004), the mouse AKAP95 protein specific to the female pronucleus (Bomar et al. 2002), or the Drosophila histone H3.3 and HIRA proteins that are only present in the male pronucleus (Loppin et al. 2005). Histone H4 is much more acetylated on paternal chromatin than maternal chromatin in mouse embryos from the time of fertilization to the first S phase (Adenot et al. 1997). Similarly, in Drosophila, acetylation of histone H4 is specific to the male pronucleus prior to the first S phase, when replication-coupled acetylation of histone H4 occurs on the maternal chromatin (Bonnefoy et al. 2007; Loppin, Bonnefoy, et al. 2005). The paternal-chromatin specific acetyl-H4 modification and localization of the histone chaperone HIRA (Loppin, Bonnefoy, et al. 2005) occur independently of YA function, and these markers could be used in future experiments to identify the male nucleus in combination with cell cycle markers to visualize the asynchronous cell cycle phenotype of *Ya* mutant embryos. The sperm nucleus incorporates maternally-provided chromatin components as it is being remodeled into the male pronucleus (Harris et al. 2003; Imschenetzky et al. 2003), yet some (e.g. histone H3.3) (Loppin, Bonnefoy, et al. 2005) are different from the maternally-provided chromatin components of the female pronucleus (canonical histone H3), and the functional significance of this distinction is unknown. A checkpoint, involving YA, for pronuclear coordination prior to the first mitosis may be advantageous in light of such distinctions between the chromatin of male and female pronuclei.

CHAPTER SIX

DISCUSSION

Egg activation has a clear beginning, upon an increase in intracellular calcium, although this calcium increase can be triggered by either fertilization (vertebrates and marine invertebrates (Ducibella et al. 2006)) or ovulation (Horner et al. 2008b). The events of egg activation are measurable molecular and cellular changes such as vitelline membrane protein crosslinking, mRNA polyadenylation, protein translation, and resumption/completion of meiosis. However, the molecular signaling pathways from the increased calcium concentration to each activation event are largely unknown. The most well-studied of these pathways are those relating to regulation of meiosis, and yet new regulators are continually being discovered, and relationships between them require further investigations. Contributing to our understanding of egg activation, I have shown that levels of all three phospho-(active) MAPKs decrease upon egg activation in Drosophila (Sackton et al. 2007, Chapter 2), representing the three major classes of MAPKs found in organisms with more repetitive proteomes. Total MAPK activity also decreases upon activation in Xenopus, mice, and marine invertebrates, as assayed biochemically (Whitaker 1996).

Regulation of MAPK signaling and other egg activation-related dephosphorylation:

The ERK-specific phosphatase MKP-3 does not have a role in egg activation, unless its role is redundant with another MKP or other dual-specificity phosphatase, because SMG translation, GNU dephosphorylation, and (probably) completion of meiosis occur in the absence of MKP3 function (in germline clones, Chapter 3). Interestingly, MKP3-null germline clone embryos have dephosphorylated ERK as in

wildtype embryos, further suggesting redundant MKP (or Tyr phosphatase) activity. The subcellular localization of MKP3 and of phospho-ERK have not been determined in Drosophila oocytes or upon egg activation, so MKP3 might be in a different subcellular compartment from phospho-ERK, and thus not acting to dephosphorylate it. Of the eight predicted MKPs in Drosophila, one (*puckered*) is specific for JNK dephosphorylation, leaving six that could dephosphorylate ERK in addition to MKP3 (Morrison et al. 2000). Because MKP3 germline clone embryos have such a clear and penetrant arrest before the first mitosis, at least one function of MKP3 is evidently not redundant with other phosphatases. This arrest phenotype is further discussed below.

As genes necessary for multiple aspects of egg activation, *cortex, sarah*, and/or *prage* might have the capacity to be regulators of MAPK or other phosphoproteins. However, none of these three genes are upstream of the dephosphorylation of ERK. In addition to their other egg activation phenotypes, I find that *cortex* (cdc20)*, sarah* (calcipressin), and *prage* are upstream of YA dephosphorylation upon egg activation, and *cortex* and *sarah*, but not *prage* are upstream of GNU dephosphorylation (Chapter 3). Figure 6.1 summarizes the interactions I have shown and their probable relationship to the initial calcium signal for egg activation.

YA, GNU, and MAPKs in oocyte maturation and egg activation:

Oocyte maturation prepares the oocyte for egg activation and early embryogenesis. Jun Cui and I have shown that the *wispy* poly(A) polymerase (PAP) (Cui et al. 2008; Benoit et al. 2008) regulates *dmos, Ya*, and *gnu* mRNA polyadenylation (Chapter 4), YA and GNU protein levels, and YA, GNU, MEK, ERK, and JNK phosphorylation (but not p38 phosphorylation). Regulation of the protein levels of DMOS, YA, and GNU by polyadenylation-dependent mRNA translation (and possibly also polyadenylation-dependent mRNA stability) downstream of a poly(A) polymerase outlines a clear pathway from *wisp* function to phenotype. Specifically,

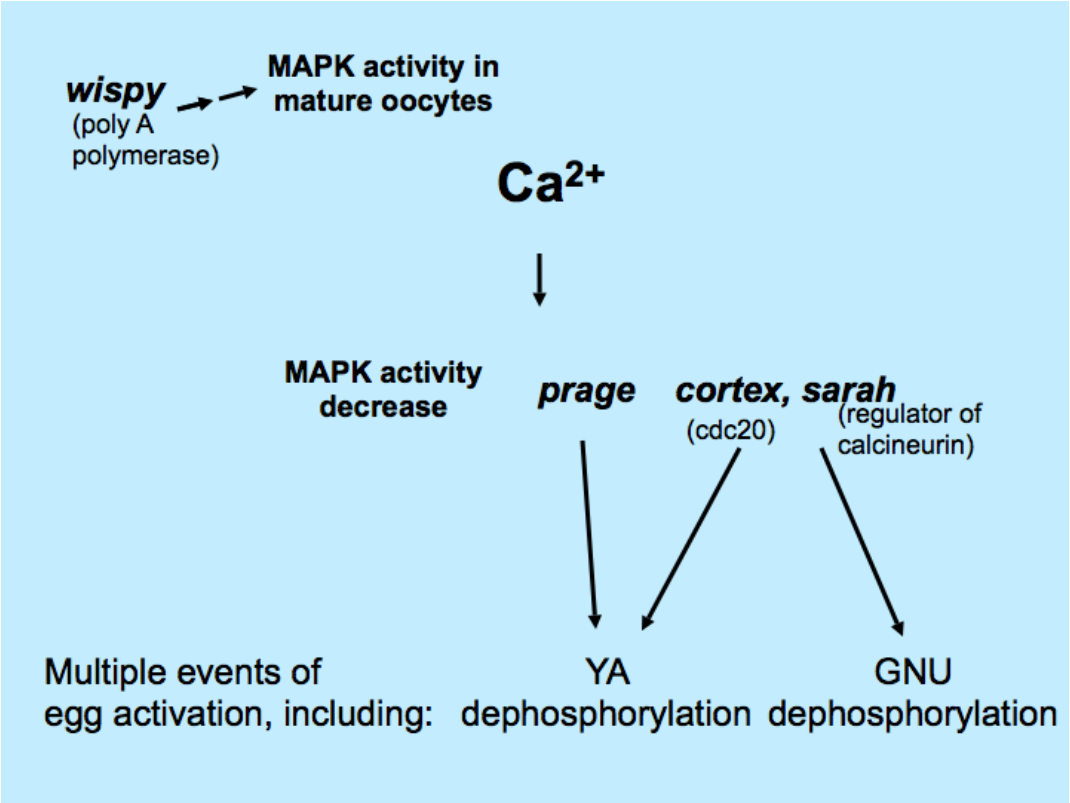


Figure 6.1 Pathways of egg activation relating to protein phosphorylation

wisp poly(A) polymerase polyadenylates *Ya*, *gnu,* and *dmos* mRNA, which increases translation of YA, GNU and DMOS proteins. Another *wisp* phenotype with a clear pathway from *wisp* function is regulation of the phosphorylation of ERK and JNK downstream of MEK downstream of DMOS which is regulated by *wisp*.

The observation that the low levels of YA and GNU protein in mature oocytes are the dephosphorylated forms of YA and GNU (the normal oocyte forms are phosphorylated) suggests that *wisp* is upstream of all the kinases that phosphorylate YA and GNU in oocytes in vivo. Loss of *wisp* activity provides an environment with both very low levels of phospho-ERK and very low levels of phospho- JNK, so these two kinases together may make a significant contribution to YA and GNU phosphorylation in oocytes. In Chapter 3 I used MAPKK germline clones to test the effect of low levels of phospho-ERK alone, or low levels of phospho-JNK and phospho-p38, but the combination found in *wisp* mutants (low phospho-ERK and low phospho-JNK) is one that I was unable to test with germline clones. For GNU, the pathway from *wisp* function to GNU phosphorylation in oocytes may be completely via MAPKs, since my preliminary results show that germline clones with very low levels of only phospho- (active) ERK have dephosphorylated GNU in oocytes, and *wisp* mutants are additionally lacking phospho- (active) JNK. Preliminary results from MEK mutants suggest both ERK and JNK are phosphorylated downstream of MEK in Drosophila oocytes (N. Buehner, preliminary data) as expected from observations in other species (Bagowski et al. 2001; Mood et al. 2004).

My mass spectrometry data suggest that YA phosphorylation in oocytes is not completely attributable to MAPKs, since only two of eleven phosphorylated amino acid residues are in MAPK consensus target sites. I hypothesize that oocyte YA in MEK mutants (very low levels of phospho-ERK and phospho-JNK) will have an intermediate gel mobility, slower than embryo YA, slower than *wisp* oocyte YA, and

faster than wildtype oocyte YA. If oocyte YA in MEK mutants is not phosphorylated on the two MAPK sites , it should migrate with similar to or faster than the intermediate mobility of T443A mutant oocyte YA, which cannot be phosphorylated on one of two MAPK sites (Yu et al. 1999).

Phosphoregulation:

Phosphorylation is a rapid enzyme-catalyzed formation of a covalent bond instead of biosynthesis of a new molecule (protein) or molecules (mRNA then protein). Consider the model of YA regulation where YA is sequestered in an oocyte cytoplasmic complex and translocates to the nucleus upon egg activation, when it is phosphorylated and released from the complex. Consistent with my protein expression data from the stages of oogenesis, showing accumulation of YA and GNU in mature oocytes (Chapter 4), this model predicts that some proteins that are not necessary in the oocyte are still translated at high levels, so that a rapid modification such as dephosphorylation is all that is needed to allow them to function early in embryo development, such as YA's function in the first mitosis. *Bicoid* and *smaug* are translated upon egg activation, which is appropriate because these proteins do not have demonstrated roles in the first mitosis or other very early (in the first minutes) events in the fertilized embryo. Of course, translation can be fairly rapid, so regulation by phosphorylation changes may not necessarily be better or faster than regulation by translation, just different. An example of rapid translation is that in transgenic flies that translate *gnu* after egg activation, GNU protein is produced rapidly enough for it to function. Specifically, a *gnu*-bcd 3'UTR transgenic construct translated upon egg activation rescues the gnu phenotype (Zhang et al. 2004). This construct functions most effectively for anterior nuclei, because it is translated in the anterior as the *bcd* patterning regulator is normally. The female meiotic products and sperm nucleus are also localized to the anterior, so even when translated after egg activation in the

gradient pattern characteristic of BCD, GNU is in the right place to regulate the first (and subsequent) mitotic cycles.

Regulating phosphoregulation:

Redundancy in phosphoregulation may make it a more robust regulatory mechanism than it would be otherwise. The MAPK kinases ERK, JNK, and p38 are redundant with respect to YA phosphorylation, although possibly not for GNU (Chapter 3). Enzymes that are redundant (one can do the work of two) might also be complementary (two work better than one), since the presence of two enzyme that can perform a function would be analogous to having more enzyme. A first model for why constitutively active ERK (rl^{Sem}) has no egg activation phenotype beyond a decrease in embryo hatchability is because perhaps ERK alone does not have as significant an effect in regulating egg activation events as a combination of MAPKs has (i.e. redundancy). A study in *C. elegans* that looked for ERK-dependent aspects of the meiosis to mitosis transition found multiple roles for ERK that were commonly shown by slight defects when ERK was perturbed alone (Lee et al. 2007). A second reason for the limited effect of rl^{Sem} in activated eggs is that a misexpressed protein cannot function in the new context if its targets are not present. With the many changes that occur between oocytes and embryos, the set of possible ERK substrates in embryos may be different from the set in oocytes. Third, ERK substrates in embryos may also not be sensitive to constitutively active ERK's presence in the new context. For example, the MAPK site phosphorylations of YA on T443 and T674 persists in embryo YA purified from 0-1hr old embryos, so when rl^{Sem} is introduced in embryos, YA is already phosphorylated on its MAPK sites and that is the state in which it functions normally, so additional kinase activity would not perturb its function. I hypothesized that rl^{Sem} would have an effect on a phosphoregulated protein that needed to be in its dephosphorylated state to function in embryos, and the primary

way its dephosphorylated state was maintained in embryos was through the absence of active ERK. The first (redundancy) model would explain the lack of phenotype from such a target if the primary way its dephosphorylated state was maintained in embryos was through the absence of all active MAPKs.

Phosphatases in egg activation:

Based on the data from Renault et al (2003) that recombinant Inhibitor-2Dm or a high concentration (but not a low concentration) of okadaic acid maintain GNU phosphorylation in oocyte extract under conditions where GNU would otherwise be dephosphorylated, I hypothesized that overexpression of Inhibitor-2Dm or Nipp *in vivo* might maintain GNU phosphorylation in embryos, where it would otherwise be dephosphorylated. However, overexpression of these two PP1 inhibitors in eggs and embryos affects overall embryo hatchability, ERK dephosphorylation, and perhaps also SMG translation, but not GNU dephosphorylation. The failure of the PP1 phosphatase inhibitors to prevent GNU dephosphorylation suggests that they did not sufficiently perturb the equilibrium between kinase and phosphatase activity acting on GNU in embryos. Decreased kinase activity could play a major role in GNU dephosphorylation, meaning low levels of phosphatase activity would suffice. There is variation in the phenotypes that are observed between inhibitors, between lines of the same inhibitor, and between Gal4 drivers, and all of these factors could affect expression levels of the inhibitors. Although we have not measured PP1 inhibitor expression levels in the various experimental treatments (comparing every line of UAS-Nipp and UAS-I2 driven by nos-Gal4 or MT-Gal4), the variation observed is most simply explained by dosage-dependence in combination with variation in levels of inhibitor. The PPI inhibitor levels may not have been high enough in any of the experimental conditions to decrease phosphatase activity below the minimum levels required to dephosphorylate GNU. My preliminary data that GNU seems to be

dephosphorylated in *dSor* germline clone oocytes suggests that decreased kinase activity is sufficient for GNU dephosphorylation in oocytes, which is consistent with the model of GNU dephosphorylation being based more on kinase inactivity than phosphatase activity. The effects that PP1 inhibitors do have may be observable because differences in phosphatase activity are more significant to the kinase/phosphatase equilibriums regulating these egg activation events. The unknown targets of PP1s that are involved in regulating SMG translation and ERK dephosphorylation seem to require more phosphatase activity, making them more easily perturbed by Nipp and Inhibitor2Dm. ERK is unlikely to be a direct PP1 target, since MAPKs are commonly dephosphorylated by dual-specificity kinases than by ser/thr kinases.

Models for YA function:

Phosphorylation is a major element of many signaling pathways, and much of the discussion in this dissertation has focused on phospho-signaling in transducing the calcium signal to various phospho-molecules that regulate aspects of egg activation. There are many examples of phospho-regulation of the cell cycle, such as the modification of cyclin-dependent kinase (cdk1) at both inhibitory and activating phosphorylation sites. The GNU and YA phosphoproteins are involved in regulating the first embryonic mitosis. The role of phosphorylation in regulating GNU function is unknown. The proposed role of oocyte-specific YA phosphorylation is to regulate YA's subcellular localization. In addition, YA has many more phosphorylated residues that are not oocyte specific, and their roles are unknown. Alanine-substitution of T443 makes YA non-functional, but it is not clear what the molecular basis for this phenotype is. Any given phosphorylation in embryos could mediate one of nuclear YA's protein-protein interactions (with lamin, H2B, or itself), or YA's interaction with DNA. There is no evidence that YA has a particular enzymatic function, so although it

is possible that it has a novel enzymatic activity that could be phosphoregulated, future experiments that perturb known phosphorylation sites should focus on testing their effects on YA's molecular interactions.

Because PCNA and H3-FLAG staining on nuclei of YA-deficient eggs and embryos suggest that S-phase can at least begin in the absence of YA function, YA may be necessary for the completion of the first S phase and/or entry into the first mitosis. Because the *Ya* embryo arrest point is not before S phase, investigations of YA's connection to the cell cycle should focus on S phase or G2-M regulators (cyclins A and B, respectively) instead of G1-S regulators (like cyclin E). However, in part because the syncytial cell cycles of early Drosophila embryos have short or no gap phases, the exact mechanisms by which cyclins exert their essential roles in controlling the syncytial embryonic cell cycles are somewhat different from mechanisms in other systems. The PanGu kinase complex, which includes GNU, is also necessary for S-phase exit and M phase entry, although in the absence of any one of the three complex components (*plu, gnu*, or *PanGu*), multiple rounds of replication occur, unlike the *Ya* phenotype. In fact, lack of YA combined with a mutation of any of these three genes prevents the multiple S phases, which suggests that YA function is not required for exit from S phase (or the mutant would display or enhance the giant nuclei phenotype). My immunofluorescence results do not support YA regulating entry into S phase because two proteins characteristic of replicating chromatin or replicated (sperm) chromatin, PCNA and canonical histone H3, are present on nuclei of YA-deficient eggs and embryos (Chapter 5). Two models are consistent with the immunofluorescence and genetic interaction data, as well as the condensed chromatin morphology of YA-arrested embryo nuclei. First, YA could act between S phase entry and exit, promoting proper S phase, and in its absence the chromatin becomes condensed instead of being in a relaxed structure that favors replication. Second, YA

could act to promote entry into the first mitosis, and when the replicated nuclei cannot enter M in the absence of YA, then become condensed. In the first model, if the chromatin condensation did not prevent epitope recognition, PCNA might be detected on the majority of condensed YA nuclei. This is not something I observed with high frequency, but there were a few instances of such nuclei.

In addition to the final arrest of YA-deficient embryos with condensed chromatin, the nuclei in such embryos can exhibit cell cycle asynchrony, although wildtype Drosophila embryo pronuclei are always synchronous, and usually the polar bodies are also synchronous with the pronuclei. The two parental genomes in the single nucleus of a mouse one-cell embryo have temporally distinct patterns of DNA methylation, low-level transcription, replication, and histone modifications such as acetylation of histone H4. (Mayer et al. 2000) In rhesus monkey zygotes fertilized by ICSI, asynchrony between the male and female pronuclei correlates with delayed onset of the first mitosis, suggesting pronuclear synchrony is the preferred state (Hewitson et al. 1999). As the Drosophila sperm nucleus is being remodeled its chromatin contains male-specific elements such as histone H3.3 (and associated chromatin regulatory factors Hira and CHD1) and acetylated histone H4 (Konev et al. 2007; Loppin, Bonnefoy, et al. 2005), although these differences do not seem to affect the cell cycle synchrony of male and female pronuclei, which replicate together when they become apposed (and the polar bodies replicate as well; all 5 nuclei are synchronous). YA may act to promote nuclear synchrony, or signal to a cell-cycle arrest checkpoint when synchrony is lost. If a protein such as YA is needed to actively promote pronuclear synchrony at apposition, then a very low frequency of wildtype asynchronous apposed pronuclei should be observed, in need of the synchronizing activity. I have not seen such wildtype nuclei, but perhaps it is not feasible to look at high enough numbers of these hard-to-obtain early embryos in order to detect an event

that occurs with very low frequency.

YA and the phosphatase MKP3 both act after egg activation, and have similar arrest phenotypes, which leads to the question: are their functions related, and if so, how? MKP3 and YA do not seem to be related through ERK, first because lack of MKP3 is not sufficient to prevent ERK dephosphorylation in embryos, and second because the two YA MAPK target site phosphorylations T443 and S674 remain phosphorylated in early embryos. That its, since the decrease of all three MAPKs upon egg activation is not sufficient to cause dephosphorylation of T443 or S674, even if lack of MKP3 caused levels of all three phospho-MAPKs to remain high in embryos (which it does not), they would not change the phosphorylation state of T443 and S674 in embryos. There are two technical issues concerning the nature of the mass spectrometry data that make this second type of data less than conclusive to say that MKP3 and YA are not related through ERK. First, although the quantification estimates suggest that levels of S674 phosphorylation do not decrease upon egg activation, and the quantification estimates for T443 suggest that it becomes phosphorylated at a lower frequency (but the percentages are highly variable), these are just estimates, not measurements, and so they are suggestive, but not conclusive. Second, the failure to detect phosphorylation of a site does not prove it is not phosphorylated. For example, if T162 were phosphorylated in oocytes but not embryos, it would fit with the role of a priming phosphorylation in GSK3-dependent phosphorylation of S158. (And if MPK3 caused abnormally high MAPK activity in embryos, which caused inappropriate T162 phosphorylation, which caused inappropriate S158 phosphorylation, that might prevent YA from entering embryo nuclei or otherwise inhibit its function, causing the arrest phenotype in MKP3 germline clones through inactivating YA, although the first point is not true, so this model is moot.) However the mass spectrometry data suggesting T162 is not

phosphorylated are actually quite strong, because peptides containing T162 were detected in most of the biological replicates, and the MS/MS spectra of ions released by individual amino acids were clean and complete, suggesting that there was excellent power to detect phosphorylation if it were there. The other MAPK consensus motif sites of YA that were not shown by mass spectrometry to be phosphorylated were each detected more than once; there are fewer T263-containing peptides than for any of the other four MAPK motif sites, but it is also the least conserved evolutionarily, which may independently suggest that this site does not have a critical, conserved function. In general, the mass spectrometry data about YA suggest that modulation of MAPK levels by MKP3 would not affect YA function, and in any case MKP3 does not appear to be essential alone for ERK dephosphorylation in embryos (Chapter 3).

If the similarity of the YA and MKP3 phenotypes were because YA was a direct MKP3 target, lack of a Tyr dephosphorylation could cause the arrest phenotype in MKP3 germline clones through inactivating YA, but there were no Tyr phosphorylations consistently detected by mass spectrometry of YA, although one of the peptides detected only once in oocytes suggested a Tyr phosphorylation on the same peptide as S674. The hypothesis that YA is a direct MKP3 substrate is unlikely, and can be tested directly by assaying for a change in YA electrophoretic mobility between control embryos and MKP3 germline clone mutant embryos (though this may be hard to detect, since there are at least nine phosphorylation sites in embryos and removal of them all by CIAP treatment causes a very slight shift, so removal of just one may not be detectable).

If the phosphatase MKP3 is not involved in phospho-regulation of YA by any of the models discussed above, perhaps it and YA have a common target, a protein that is regulated both by phosphorylation change and by interaction with YA. If my

ongoing experiment to look for proteins that interact with YA in embryos identifies a protein with a predicted Tyr phosphorylation, that protein would be a candidate for testing this model for how the YA and MKP3 embryo phenotypes are related. The MKP3 arrest should also be characterized by looking for lamin and YA localization to the nuclear lamina of the MKP3 germline clone embryo's arrested nuclei.

My investigations of phosphoregulation at the oocyte to embryo transition have related to many different proteins active at this time. These included the egg activation regulators SRA, CORT, and PRG and WISP, which regulates aspects of both oocyte maturation and egg activation. I also experimented with proteins of various MAP kinase cascade, and considered the roles of other kinases acting at this time when interpreting my results. My experiments with phosphatases included both ser/thr phosphatases (PP1s) and a dual-specificity phosphatase (MKP3). I assayed the phosphorylation of YA and GNU, two phosphoproteins that regulate the first embryonic division. With all these various proteins assayed for their roles immediately before, during, or immediately after egg activation, there are still many more candidates to investigate. My data suggests the importance of phosphorylation in connecting calcium to egg activation and for the first mitosis. I have contributed to the eventual understanding of how the calcium increase signals the events of egg activation, and how the unique cell cycle of the first mitosis is regulated.

APPENDIX A

YA HOMOLOGS AND COMPLEMENTATION TESTS

A.1 Introduction

The null mutant alleles Ya^{2} and Ya^{76} prevent the fertilized egg from beginning mitosis and cause an abnormally condensed chromatin state in the haploid nuclei (Lin et al. 1991). Despite conservation of this critical time point in development across animals, no homologs of YA outside of Drosophila have previously been found. I aligned YA sequences from 8-13 Drosophila species to identify conserved functional regions (for a phylogenetic tree of Drosophila, see flybase.bio.indiana.edu/blast/). This search uncovered a conserved cysteine/histidine rich region (likely involved in protein-protein or protein-DNA binding), as well as distinguishing the more conserved three of the five MAPK phosphorylation sites. I have shown by mass spectrometry that two of these conserved sites sites are phosphorylated in oocytes and embryos (Chapter 3).

Using the YA cysteine/histidine rich region to BLAST Genbank, I found the mouse protein Zar1. Like YA, the mouse Zar1 null is a female sterile mutation. Most zygotes that lack maternal Zar1 arrest before the first division, and less than 20% reach the two cell stage (Wu, Viveiros, et al. 2003). This phenotype is similar to the arrest point of YA, although, like YA, the molecular function of Zar1 remains to be determined.

Several genes have been identified in Drosophila screens as necessary for egg activation or early embryonic mitosis, and many of those with cloned gene sequences have orthologs in other species (*wisp*, *sra*, and *cort*,)(Cui et al. 2008; Horner et al. 2006; Swan et al. 2007). However, there are no homologs detected by BLAST outside

the Drosophila genus for *plu, gnu, PanGu*, or *ms(3)K81* (Lee et al. 2003; Loppin, Lepetit, et al. 2005). I have identified putative orthologs for *Ya* by reciprocal BLAST searches in the mosquitos *Anopheles gambiae*, *Aedes aegypti*, and *Culex pipiens quinquefasciatus*, which are dipterans like Drosophila. Bioinformatic searches and degenerate PCR have not identified *Ya* homologs in other insect orders such as hymenoptera.

A.2 Materials and Methods

Multiple sequences were aligned by the Clustal W method in Megalign, with default alignment gap opening and gap extension penalties. Conserved amino acid sequences from the alignment of D. melanogaster, *D. sechellia*, and *D. pseudoobscura* were used for tBLASTn against the genomes of other Drosophila and other insect species.

Degenerate PCR: I designed degenerate PCR primers to regions of conserved amino acid sequence between *D. melanogaster, D. sechellia*, and *D. pseudoobscura*, the three species whose entire genome sequences were publicly available at the time of primer design. As the *D. melanogaster Ya* coding sequence is 2091bp long, I designed nine primers to four areas of YA: the two ends and two relatively conserved areas near the middle. I made the primer sequence degenerate wherever *D. pseudoobsura* and *D. melanogaster* differed, usually at the third position, but in other positions where necessary in some cases. PCR was done using promega Taq and either standard denaturing/annealing cycles or touchdown PCR (multiple annealing temperatures used sequentially).

Western Blots: Samples were prepared for gel electrophoresis by boiling in sample-buffer for 3 minutes, and protein from 1-2 females was loaded into each lane of a 7.5% SDS polyacrylamide gel. Western blots were prepared as previously described (Monsma et al. 1988). Anti-FLAG M2 (Sigma) was diluted 1:1000. Anti-YA raised against the C-term of YA (J. Liu et al. 1995) was diluted 1:1,000. Two other anti-YA

antibodies, raised against the N-term and middle of YA (N. Buehner) were also used, and they also recognized D. sechellia YA but not D. virilis YA.

Transgenic flies: UASp-YA and UASp-FLAG-YA with YA from *D. sechellia* or *D. virilis* were generated by cloning the *Ya* full-length cDNA coding sequence from these species into Gateway (Invitrogen, Carlsbad, CA) *P*-element transformation vectors, one of which includes the 3X-FLAG sequence N-terminal to the Gateway insertion site (gift of T. Murphy, Carnegie Institute of Washington, MD). UASp-Zar1 and UASp-FLAG Zar1 were generated by the same method after cloning the Zar1 full-length cDNA coding sequence by PCR of exons 2-4 from mouse cDNA and PCR of exon 1 from a genomic BAC plasmid (RP23-384C22, BACPAC.chori.org), followed by blunt-end ligation to make the full-length Zar1 CDS. Transgenic lines were generated by injecting DNA for germline transformation into embryos of the w^{1118} strain of *D. melanogaster* using standard procedures (Rubin et al. 1982).

Complementation Tests: The apparent null allele Ya^2 (Lin et al. 1991) , is carried in stock females that also carry the *nanos-Gal4* driver (Van Doren et al. 1998) as ***X^X, Ya^2 / Y, Ya^+;nanos-Gal4.*** (This line was made by N. Buehner.) When these females are crossed to w^{1118} or UAS-transgenic males, they will be infertile due to the null allele Ya^2 unless the transgene expressed under UAS-Gal4 control provides functional YA.

A.3 Results and Discussion

Sequence Analysis:

The protein-coding sequences of YA from thirteen Drosophila species range in length from 687 to 762 amino acids long (Figure A.1). The C2H2 Zinc finger has the critical two cysteines and two histidines conserved in all 13 species, within a 26 amino acid region of high similarity. The serine that is mutated in the Ya^{76} putative null allele is conserved in 12 species and is a threonine in *D. anannasse*; it is located in a region of 12 highly similar amino acids. The C-terminal basic-residue rich region has at least

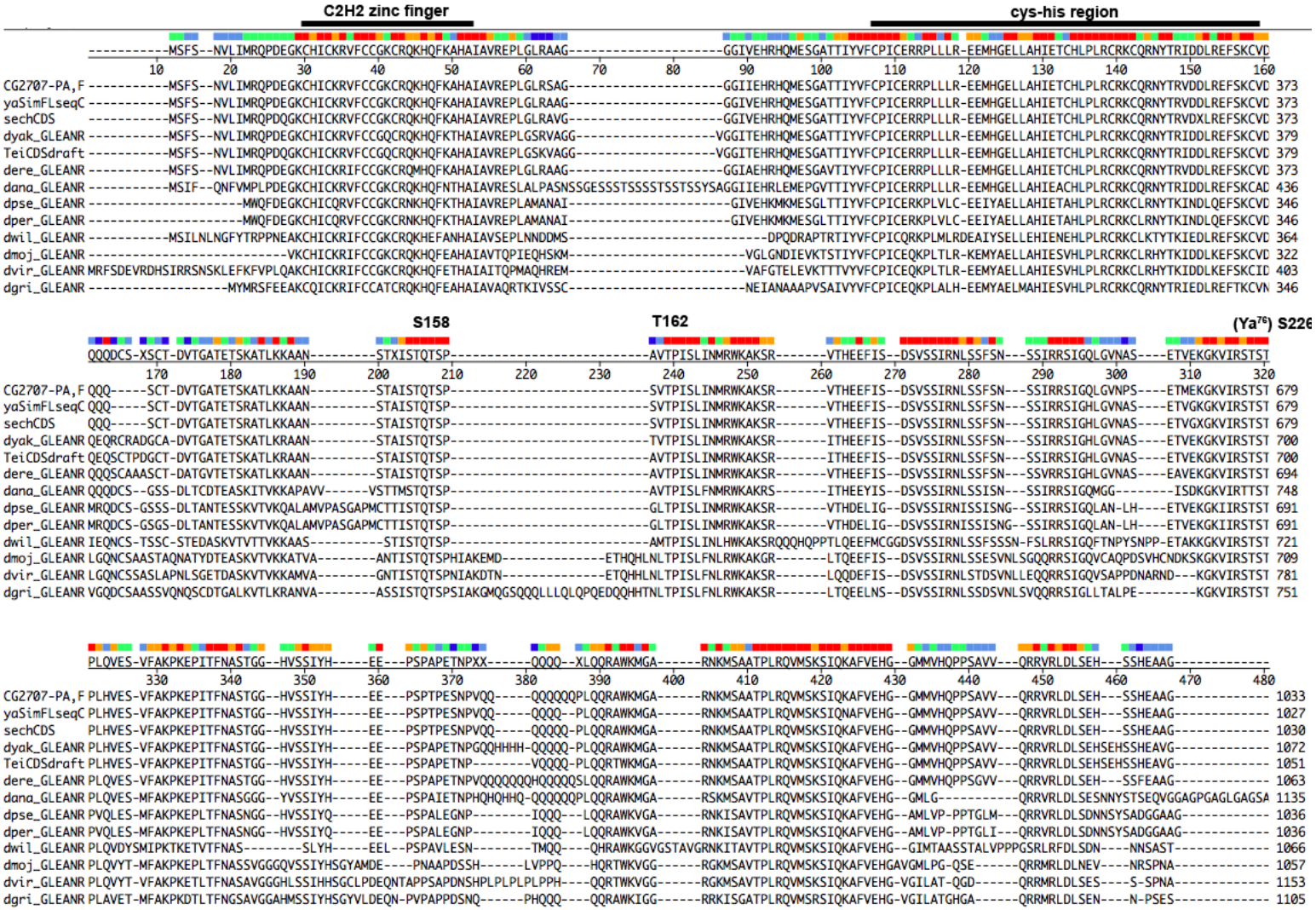
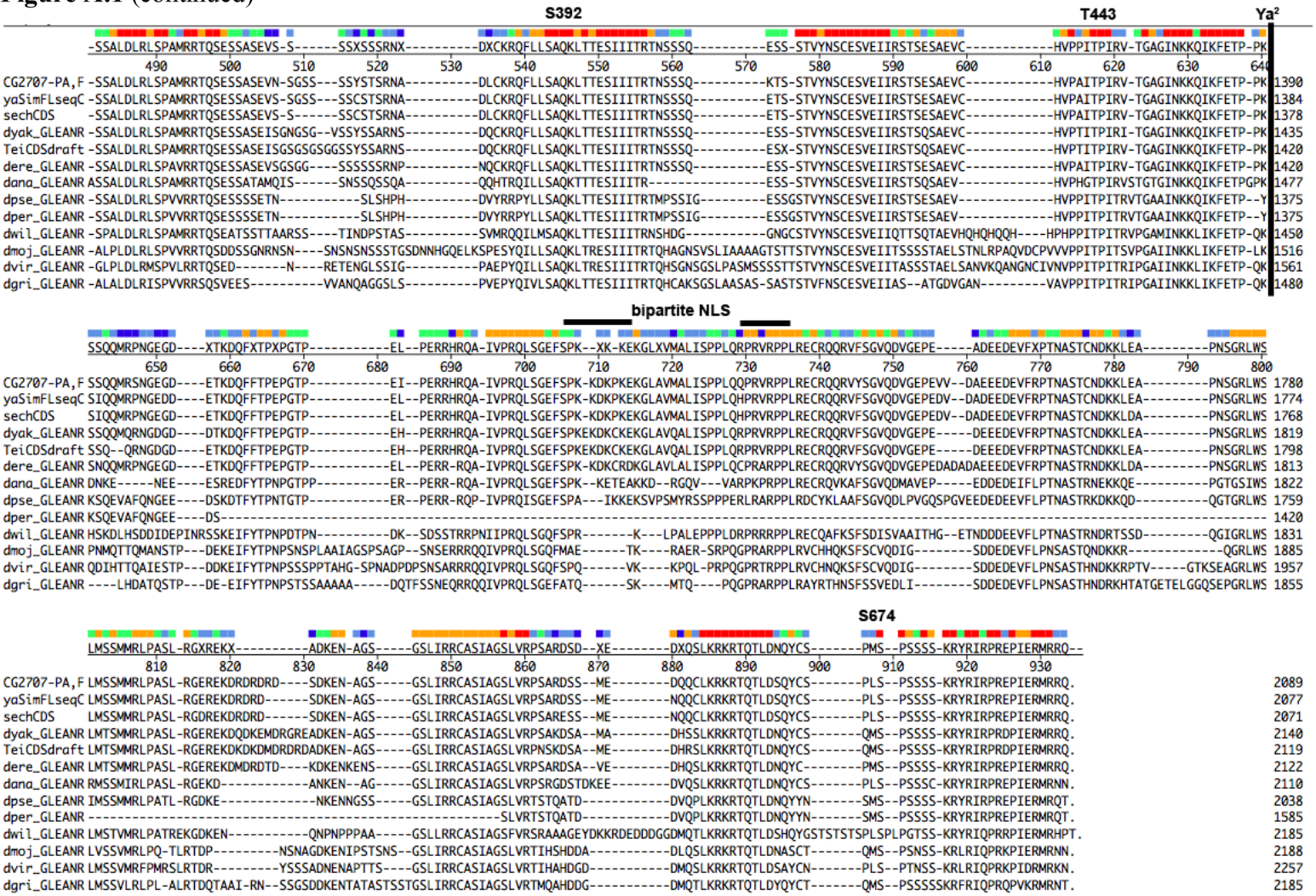


Figure A.1 Alignment of YA amino acid sequence from thirteen Drosophila species

Figure A.1 (continued)



60% of the positively charged residues in D. melanogaster in all 13 species. At MAPK site T443, all three relevant target motif amino acids (prolines and threonine) are completely conserved. At MAPK site S674 the SP is completely conserved, but the upstream proline is only present in 7/13 species, and these seven are not the species closest to melanogaster (suggesting loss in yakuba, teissieri, pseudoobscura, persimilis, mojavensis, and grimshawi). The GSK3 site at S158 and the MAPK site at T162 (which should be phosphorylated as part of the GSK3 target motif) have a large (14-28 amino acid) insertion between them in virilis, mojavensis, and grimshawi that could disrupt recognition.

Zar1, a potential functional homolog of YA:

The Zar1 and YA whole gene sequences cannot be aligned, but both have a conserved cystine/histidine rich region. This region has eight cysteine and histidine residues, and the spacing between them is conserved in sequences of Zar1 from six vertebrate species (Wu, Wang, et al. 2003). The spacing of the eight cysteines and histidines is also conserved between YA sequences from twelve (*D. willistoni* has a one aa insertion between C2 and H3) Drosophila species, but the spacing is different between mouse and fly (Figure A.2). The cystine/histidine region is near a C2H2 zinc finger at the amino terminus of YA, and at the carboxy terminus of Zar1 (Liu et al. 1998; Wu, Wang, et al. 2003). The C2H2 zinc finger and the bipartite NLS are the only well-defined sequence motifs in YA, and do not define a proposed molecular function (except DNA binding). In fact, the predicted NLS at YA's C-term does not precisely fit any of the the NLS consensus motifs used at the predictNLS server website (Cokol et al. 2000), but seems to function for YA localization. Although the sequence of YA is novel, its structure may be similar to the structure of the analogous protein in other organisms. As increasing numbers of protein structures are solved, other proteins with conserved functions but not sequence have been identified. For

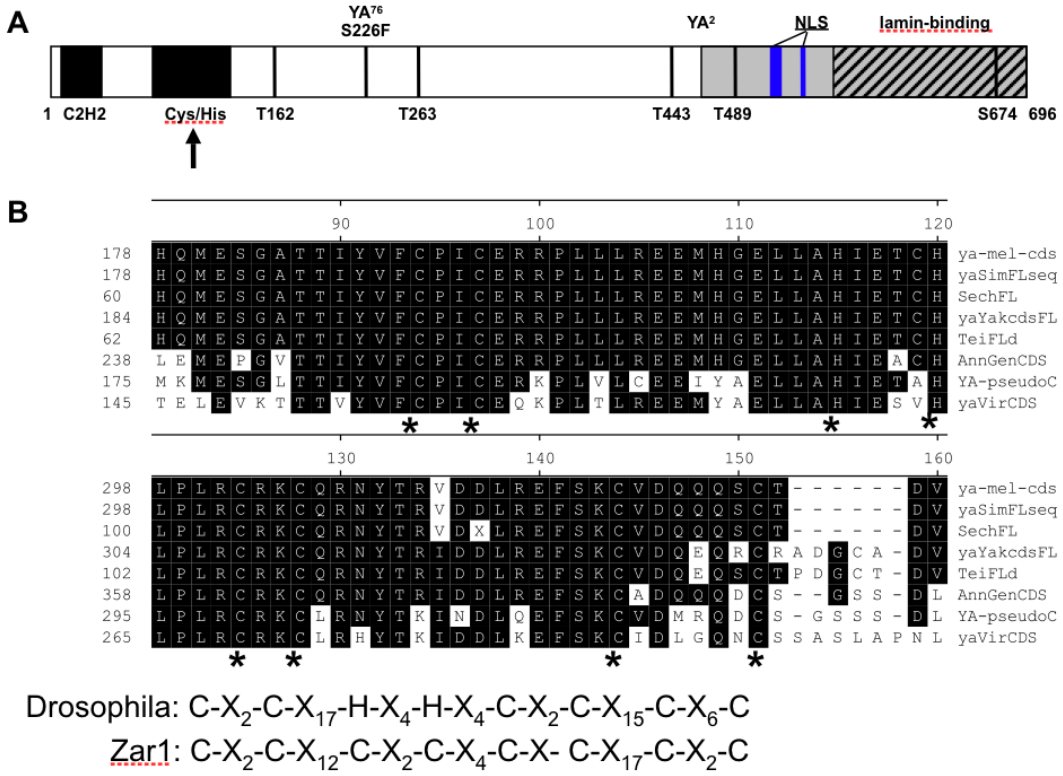


Figure A.2 Cysteine/histidine rich region

(A) Diagram of YA protein with features indicated. Arrow points to cysteine/histidine rich region. (B) Alignment of the cysteine/histidine rich region of YA from eight Drosophila species and comparison of the spacing with that of the cysteine/histidine rich region of Zar1.

example, the prokaryotic protein ThiS and eukaryotic ubiquitin fold similarly and share a sulfur-binding active site, with only 14% sequence identity (Wang et al. 2001). Likewise, the reproductive proteins Sp18 and lysin have similar structures and mediate sperm-egg interactions in two subsequent steps, yet have only 18% identity (Kresge et al. 2001).

I used the Robetta structural prediction program to generate sets of theoretical structures for the cystine/histidine rich regions of YA and Zar1 (Kim et al. 2004). I also tested whether these regions were predicted to be similar to the same types of protein-folding motifs when submitted to the Protein Structure MetaServer 3D-Jury (Ginalski et al. 2003) and Foldminer (Shapiro et al. 2004). This resulted in lists of fold-motifs for the YA cystine/histidine rich region and for the Zar1 cystine/histidine rich region with some fold-motifs in common. I was unable to determine whether the similarities between the lists were more significant for YA and Zar1's cystine/histidine rich regions than would occur randomly, so there was no significant result of these structural comparison attempts.

Degenerate PCR:

I designed degenerate PCR primers to regions of conserved amino acid sequence between *D. melanogaster, D. sechellia*, and *D. pseudoobscura*. I made cDNA from RNA isolated from several Drosophila species and *Culex pipiens quinquefasciatus* (the mosquito C. pipiens) and *Nasonia vitripennis* (a small wasp). Neither these templates nor *D. ananassae* cDNA produced any PCR products (Figure A.3 and data not shown), but the cDNA from several Drosophila species, including *D. lutescans* and *D. eugracilis*, did produce some PCR products of the expected sizes which could be purified and sequenced.

BLAST searches:

Using full length YA and conserved regions of YA, I ran tBLASTn against the

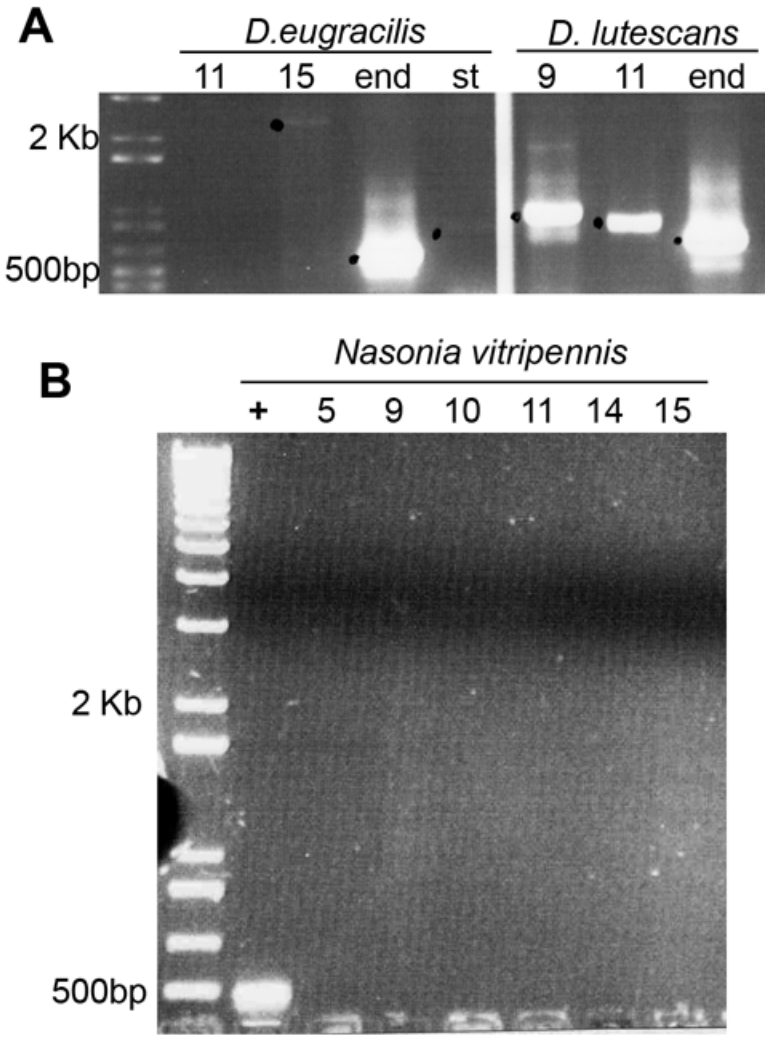


Figure A.3 Degenerate PCR products

Degenerate PCR from ovary RNA of D. eugracilis, D. lutescans, and whole female RNA of Nasonia Vitripennis. (A) Primer pairs that amplify various regions of *Ya* worked to different degrees in different Drosophila species. (B) Primer pairs that amplify various regions of *Ya* did not produce products in Nasonia, although a highly conserved housekeeping gene did produce a product, verifying cDNA quality.

A. gambiae, A. aegypti, and *C. pipiens* mosquito genomes, as well as the *B. mori* (silkworm), *Apis mellifera* (honey bee), *N. vitripennis* (wasp), and *Tribolium castaneum* (flour beetle) genomes. In all three mosquito genomes I found significant alignments to full-length YA. When the predicted amino acid sequences of *A. gambiae* transcript AGAP003740-RA, *C. pipiens* transcript (BROAD cds) BDAG_11448, and the *A. aegypti* transcript AAEL007813-RA are submitted for tBLASTn against *D. melanogaster, Ya* is the best hit, and the three mosquito genes are also the best reciprocal BLAST hits for each other. Therefore, these are likely mosquito YA homologs. However, neither full length YA or conserved regions of YA found significant matches in the other insect genomes. Therefore, YA is conserved among dipterans, but may not be recognizable in other insect orders.

Complementation Testing:

As controls, I made transgenic flies expressing *D. melanogaster* (mel) YA. UAS-mel YA or UAS-FLAG-melYA can rescue the Ya^2 mutant infertility phenotype when driven by *nanos*-GAL4 in the Ya^2 mutant background. To test the functional conservation of YA in other Drosophila species, I made transgenic flies carrying YA from a species closely related to *D. melanogaster*, *D. sechellia* (UAS-sechYA, UAS-FLAG-sechYA), and to the more distantly-related species *D. virilis* (UAS-virYA). To test for expression of VirYA, which is not recognized by the YA antibodies raised against melYA (Figure A.4), I isolated RNA from the Ya^2;virYA infertile females, and did RT-PCR using virYA-specific primers. I found that simYA and virYA were expressed.

To test whether Zar1, although mostly unlike YA in primary sequence, could complement the Ya^2 null mutant phenotype, I made transgenic flies carrying Zar1 (UAS-Zar1, UAS-FLAG-Zar1). Expression of FLAG-Zar1 was confirmed by anti-FLAG western blot. *D. sechellia* YA, which has 95% protein identity with

melanogaster YA, rescued the Ya^2 mutant infertility phenotype, but *D. virilis* YA, which has only 51% protein identity with *D. melanogaster* YA, did not rescue (Table A.1). The *Drosophila virilis* YA homolog has reasonable sequence identity with *D. melanogaster* YA, and presumably performs the same function in its own species, yet it could not function in *D. melanogaster.* Thus the result that Zar1 could not rescue the Ya^2 null mutant phenotype does not mean that it might not perform a similar function to YA's in its own species, as suggested by the arrest of mouse embryos mutant for Zar1, but because it does not complement, there is no genetic evidence that it is a functional YA homolog.

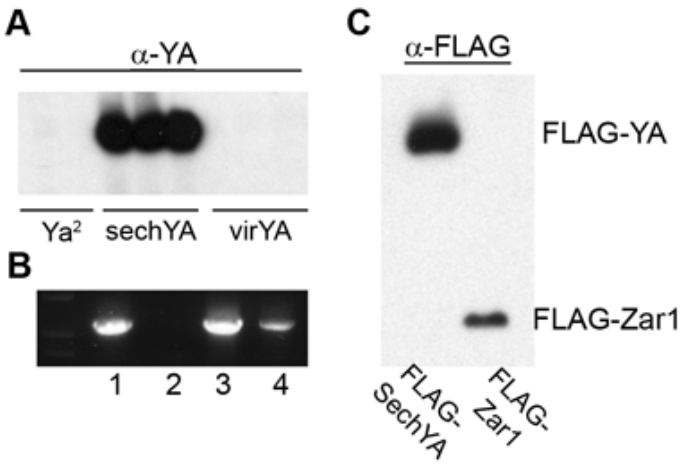


Figure A.4 Transgenic YA and Zar1 expression

(A, C) Western blots using anti-FLAG and anti-YA antibodies on samples from ovaries of Ya^2;UAS-transgenic;nos-Gal4 or Ya^2 (negative control) females. (A) Anti-YA raised against *D. melanogaster* recognized *D. sechellia* YA but not *D. virilis* YA. (B) RT-PCR of RNA from (1) *D.virilis* female ovaries (positive control), and (2-4) RNA from ovaries of daughters of three lines of FLAG-virYA crossed to Ya^2;nos-Gal4. (C) Anti-FLAG blot shows expression of FLAG-Sech YA and FLAG-Zar1.

Table A.1 Results of Complementation tests

	number of lines	complement *Ya*^{2}	protein expressed
UAS-sech YA	3	**yes**	yes, anti-YA
UAS-FLAG-sechYA	2	**yes**	yes, anti-FLAG
UAS-virilis YA	3/5	no	RT-PCR 3/5 yes
UAS-Zar1	4	no	NT
UAS-FLAG-Zar1	2	no	yes, anti-FLAG
W1118	1	no	NT

APPENDIX B

NUCLEAR ENVELOPE PROTEIN INTERACTORS

B.1 Introduction

In embryos, YA protein localizes to the nuclear lamina and nucleoplasm (Lopez et al. 1994). In yeast two-hybrid studies, YA interacts with the Drosophila B-type lamin, lamin Dm0, and lamin interacts with otefin, another Drosophila nuclear lamina protein (Goldberg et al. 1998). Otefin is a LEM-domain (LAP2, emerin, MAN1) containing protein, as is Man1, and they both interact with BAF (barrier-to-autointegration factor) (reviewed in Gruenbaum et al. 2005). Using fractionation of mouse tissue culture cells, Dreger et al identified a set of novel inner nuclear membrane proteins (Dreger et al. 2001), orthologs of which we identified in Drosophila via a bioinformatics approach. After working with undergraduate Carissa Perez and rotation student Moses Ong on cloning these genes into the Matchmaker yeast two-hybrid system, I completed the testing of interactions between the nuclear lamina proteins YA, lamin, otefin, BAF, and Man1 and nine candidate Drosophila inner nuclear membrane proteins.

B.2 Materials and Methods

Lamina genes tested: YA (CG2707), lamin Dm0 (CG6944), otefin (CG5581), BAF (CG7380), Man1 (CG3167). Candidate inner nuclear membrane genes tested: Hel25 (CG7269), p68 Hel (CG10077), "pHel" (CG11092), PKA (CG4379), Rack1 (CG7111), Nurim (CG7655), Nup98 (CG10198), Nup 154 (CG4579), Calnexin (CG11958). All genes were PCR amplified from cDNA and cloned into Gateway pENTR-D-Topo entry vectors. LR reactions were performed to transfer each gene into Matchmaker Y2H (Clontech, Mountain View, CA) Gateway destination vectors

for both the DNA-binding domain vector pGBKT7 and the activation domain vector pGADT7. I transformed AD-fusion and BD-fusion vectors into yeast strains AH109 (mating type a), and Y187 (mating type alpha). To test for protein-protein interactions, AD-fusions and BD-fusions in complementary mating type strains were mated in liquid culture, and plated on selective (nutritional dropout) media. Diploid progeny of successful matings containing both genes grew on double dropout (-Trp -Leu) synthetic medium, and interactions were shown by growth on triple (-Trp -Leu –His) or quadruple (-Trp -Leu -His –Ade) dropout plates. Lamin, YA, BAF, Man1 and Otefin were tested for interaction with pools that did not contain known interactors and separately tested for known interactions.

B.3 Results and Discussion

Our positive controls confirmed that YA interacts with lamin and lamin interacts with otefin (Goldberg et al. 1998). The two new interactions detected were interactions between lamin and p68 hel (CG10077) and between lamin and "pHel" (CG11092). Several interactions suggested by previous research did not occur in this yeast two hybrid system, such as otefin and BAF (inferred from LEM domain), BAF and Man1 (Liu et al. 2003), lamin and Man1 (Wagner et al. 2006), and PKA and RACK. Similarly, none of these interactions occurred in the yeast two-hybrid screen of the Drosophila proteome done by (Giot et al. 2003). That screen did not detect the lamin and CG11092 interaction, although it did detect thirteen other CG11092 interactors. Yeast two hybrid commonly gives false negative results, perhaps due to low protein expression or to protein folding defects. We tested whether PKA and RACK interacted with each other, although neither of them is a known nuclear lamina protein in Drosophila. PKA and RACK may not directly interact, but RACK and PKC do interact, and there is crosstalk between PKA and PKC signaling pathways (Yao et al. 2008). However, RACK and PKA do not interact by yeast two hybrid. Of the two

new putative lamin interactors, p68 hel (CG10077) is an RNA helicase, and CG11092 is a gene of unknown function that was at some point erroneously identified as the homolog of a helicase identified in Dreger et al's fractionation experiment. A p68 RNA helicase that interacts with DNA has been purified from mouse nuclear matrix (Enukashvily et al. 2005), suggesting that the interaction we detected by yeast-two hybrid is possible.

Table B.1 Yeast-2-Hybrid results: Lamin (A), YA (B), Otefin (C), Man1 and BAF were tested for interactions with all the genes assayed in both directions (activation domain/binding domain). Man1 and BAF did not interact with any of the genes tested.

A

gene	gene	BD/AD interaction	AD/BD interaction
lamin	YA	no	yes
lamin	lamin	yes	yes
lamin	otefin	yes	yes
lamin	BAF	no	no
lamin	Man1	no	no
lamin	pHel	yes	Poor diploid growth
lamin	Hel25	no	no
lamin	PKA	no	no
lamin	Rack	no	no
lamin	Nurim	no	no
lamin	Nup98	no	
lamin	Nup154	no	no
lamin	Calnexin	no	no
lamin	p68 helicase	yes	Poor diploid growth

B

gene	gene	BD/AD interaction	AD/BD interaction
YA	YA	no	no
YA	lamin	yes	no
YA	otefin	no	no
YA	BAF	no	no
YA	Man1	no	no
YA	pHel	no	no
YA	Hel25	no	no
YA	PKA	no	no
YA	Rack	no	no
YA	Nurim	no	no
YA	Nup98	no	
YA	Nup154	no	no
YA	Calnexin	no	no
YA	p68 helicase	no	no

C

gene	gene	BD/AD interaction	AD/BD interaction
otefin	YA	no	no
otefin	lamin	yes	yes
otefin	otefin	no	no
otefin	BAF	no	no
otefin	Man1	no	no
otefin	pHel	no	no
otefin	Hel25	no	no
otefin	PKA	no	no
otefin	Rack	no	no
otefin	Nurim	no	no
otefin	Nup98	no	
otefin	Nup154	no	no
otefin	Calnexin	no	no
otefin	p68 helicase	no	no

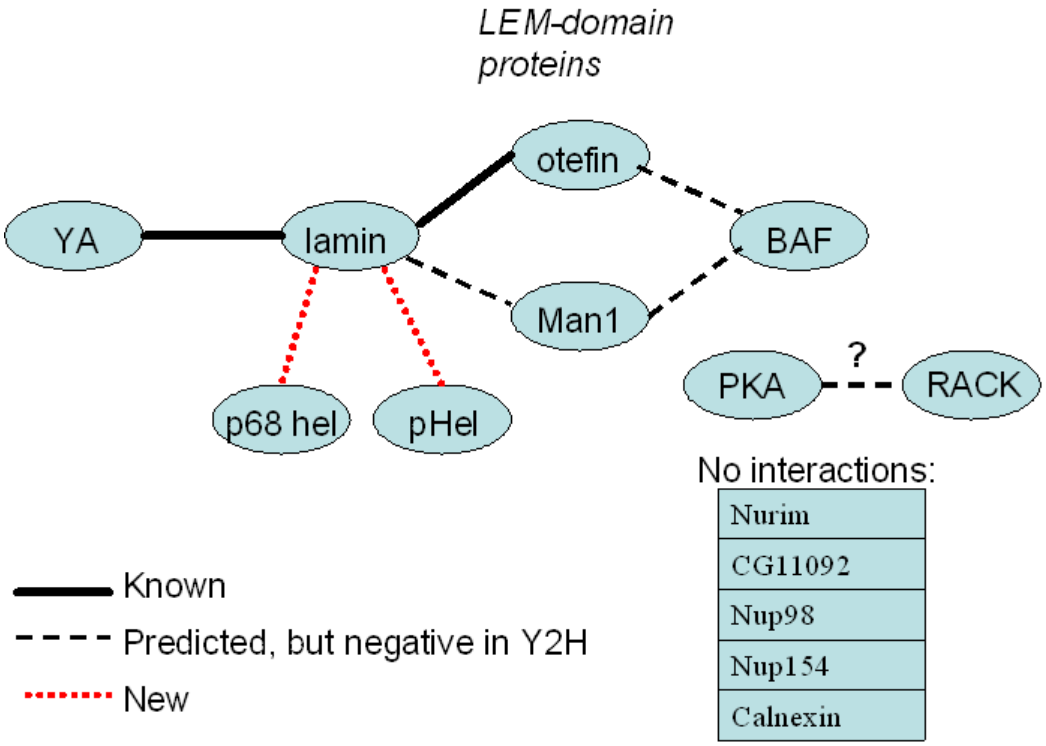


Figure B.1 Nuclear lamina yeast-2-hybrid summary and comparison:

Between the fourteen genes tested, two known interactions were confirmed (black solid lines), four known interactions were not detected (black dashed lines), and two new interactions were identified (red dotted lines). The five genes with no predicted or detected interactions are indicated: Nurim, Nup98, Nup154, Calnexin, and CG10077.

APPENDIX C

CHANGES IN MAPK LEVELS UPON IN VITRO ACTIVATION

C.1 Introduction

Egg activation in Drosophila is independent of fertilization, and can be triggered in vitro by incubation of mature oocytes in a hypotonic solution that causes the oocytes to swell as they typically do in the female reproductive tract during ovulation (Heifetz et al. 2001; Page et al. 1997). Many aspects of egg activation occur normally upon in vitro activation; meiosis resumes and completes, mRNAs are polyadenylated and translated. However, compared to laid, unfertilized eggs which are activated in vivo, in vitro activated eggs do not undergo the characteristic nuclear morphology changes after completion of meiosis (Page et al. 1997). In vitro activated eggs also do not destabilize those maternal transcripts that are normally degraded upon egg activation (Tadros et al. 2003). I tested whether the decrease in levels of phospho- (active) MAPKs upon egg activation occurs in eggs activated in vitro. I also assayed the extent of GNU dephosphorylation upon in vitro activation.

C.2 Materials and Methods

Wild type ORP2 oocytes (primarily stage 14) were isolated by the blender method (Page et al. 1997) and either left on ice or frozen in liquid N2 for an oocyte sample, or activated for 25 minutes in hypotonic Activation Buffer. Samples were ground in PIPIHB (Sackton et al. 2007) and Sample Buffer was added 1:1. Western blots for total ERK, phospho-ERK, phospho-p38, were performed as in (Sackton et al. 2007). Anti-GNU, a gift of T. Orr-Weaver was used at 1:5,000 dilution (Lee et al. 2003).

C.3 Results and Discussion

There is variation from egg to egg in the rate of response to in vitro activation. For example, when mature oocytes dissected out of the ovaries are washed into activation buffer at the same time, and then fixed and examined after various lengths of incubation in activation buffer, their meiotic progression is not synchronous. At each time point there is a distribution of meiotic stages (Page et al. 1997). The dephosphorylation of ERK upon egg activation occurred to varying extents in different samples representing pools of about 100 eggs activated for 25 minutes before being dechorionated in 50% bleach for 2 minutes and then homogenized for western blot analysis. Levels of phospho-ERK relative to total ERK were quantified, and these levels were sometimes higher than laid activated eggs and embryos, but sometimes equally as low as laid eggs/embryos (Fig C.1). Levels of phospho-p38 relative to total ERK were quantified, and these levels were either as high as oocytes or higher.

The variation in phospho-MAPK levels correlated with variation in bleach resistance. The eggs lysed in bleach were mostly not included in the samples, so there may be other aspects of egg activation affected in the bleach-resistant eggs that relate to the phospho-MAPK levels. The activation batches that demonstrated low bleach resistance were also inefficient at ERK dephosphorylation, and as percent bleach resistance increased, phospho-ERK levels decreased. I assume the activation batches where there was high bleach resistance were more efficiently activated in other aspects as well. Thus when activation is more efficient, ERK is dephosphorylated more efficiently, and this correlation is statistically significant by one-way ANOVA, $F_{(1,22)}=11.538$, $p=0.0026$ (Fig C.2). Therefore, in general ERK is dephosphorylated upon in vitro egg activation as in vivo. However, although the activation batches with low bleach resistance had relatively unchanged levels of phospho-p38 (similar to unchanged levels of phospho-ERK), as percent bleach resistance increased, phospho-

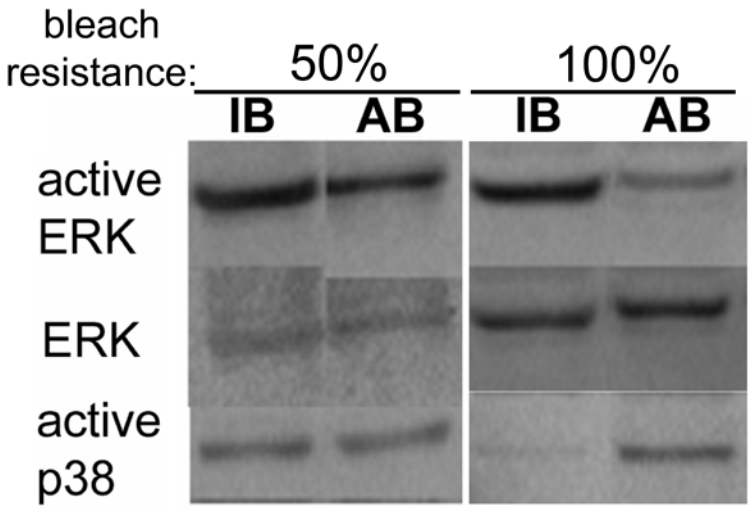


Figure C.1 Phospho-ERK and phospho-p38 levels upon in vitro egg activation

IB=Isolation buffer, AB=Activation buffer. When bleach resistance is low (left two columns), both phospho-ERK and phospho-p38 levels upon in vitro activation stay similar to unactivated (IB) oocytes. When bleach resistance is high (right two columns), phospho-ERK levels decrease and phospho-p38 levels increase upon in vitro activation. Blots shown represent part of the spectrum of variation observed in six (p38) or seven (ERK) independent experiments.

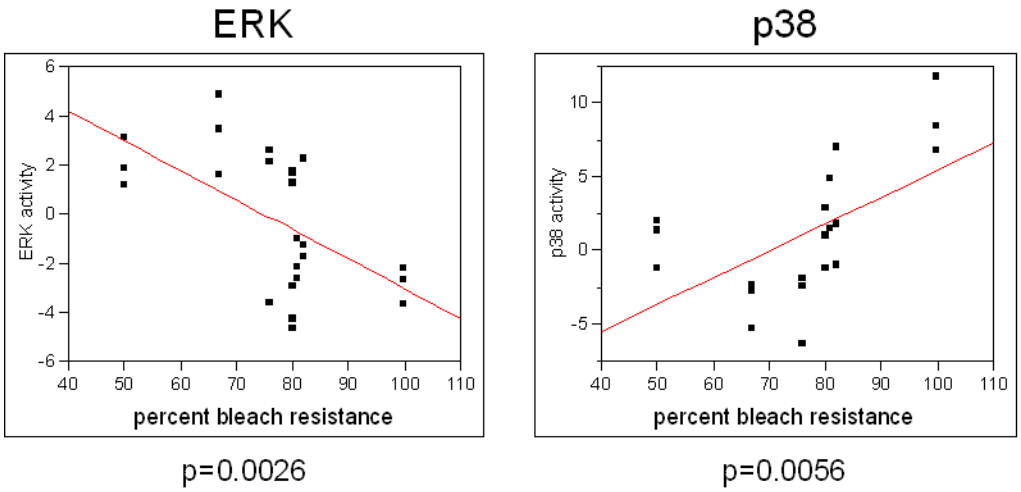


Figure C.2 MAPK activity correlates with percent bleach resistance

Each point represents a gel band quantified relative to total ERK loading in six (p38) or seven (ERK) independent experiments. The p-value of an ANOVA testing for correlation is indicated below each graph.

p38 levels increased. Thus when activation is more efficient, p38 behaves counter to its in vivo phosphorylation pattern, and this correlation is statistically significant by one-way ANOVA, $F_{(1,19)}$=9.740, p=0.0056 (Fig C.2). Therefore in general p38 is phosphorylated upon in vitro egg activation, contrary to in vivo egg activation. There is some precedent for the increase in p38 in activation buffer: p38 responds to osmotic stress in other systems (Johnson et al. 2002; Niswander et al. 2007), although it is usually hyperosmotic stress, and activation buffer is hypotonic. The variation observed in phospho-JNK levels did not correlate with bleach resistance, or follow any other discernible pattern (data not shown). Because the MAPKs do not all behave normally upon in vitro egg activation, and there is huge variation in the results, experiments adding MAPK-inhibiting drugs to activation buffer would be uninterpretable.

I also assayed for GNU dephosphorylation upon in vitro egg activation. GNU was present in its slower electrophoretic mobility form in oocytes dissected in isolation buffer, consistent with it normally being phosphorylated in oocytes. (Fig C.3) GNU's electrophoretic mobility was variable between in vitro activations. The phosphorylation state of GNU as assessed by gel mobility is difficult to quantify because there are multiple bands per lane, and sometimes smears. However the trend in GNU phosphorylation upon in vitro egg activation relative to percent bleach resistance is evident without quantification. From activations with lower bleach resistance, slow-mobility GNU was still present upon in vitro activation, reflecting lack of dephosphorylation. However, in activations with higher bleach resistance, the GNU from in vitro activated eggs was primarily the faster-mobility, dephosphorylated form. Therefore GNU is dephosphorylated upon in vitro egg activation.

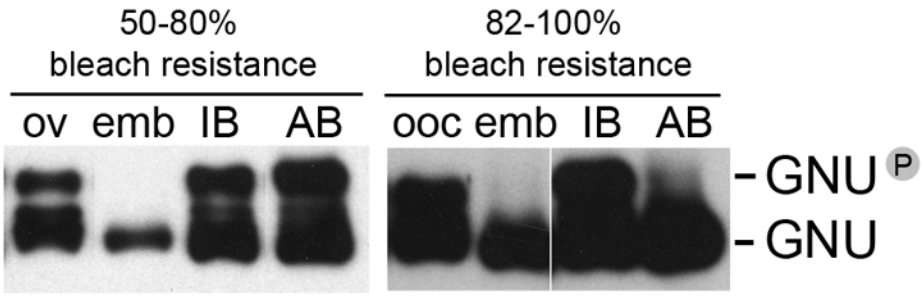


Figure C.3 GNU is dephosphorylated upon in vitro egg activation

ooc=stage 14 mature oocytes, emb=0-1hour embryo collection, IB=Isolation buffer, AB=Activation buffer. When bleach resistance is low (left-hand blot), GNU has similar electrophoretic mobility after incubation in activation buffer to its mobility in oocytes. When bleach resistance is high (right-hand blot), GNU has faster electrophoretic mobility after incubation in activation buffer than in oocytes, as is the case for embryo GNU. Blots shown represent part of the spectrum of variation observed in five independent experiments, and each is representative of the 2-3 blots performed at the range of bleach resistance indicated at the top.

APPENDIX D

DNA REPLICATION IN INDIVIDUAL MEIOTIC PRODUCTS CANNOT BE VISUALIZED BY BRDU INCORPORATION

D.1 Introduction

Because YA may act to regulate the cell cycle at the transition from meiosis to mitosis, defining the cell cycle arrest of YA mutant embryos as accurately as possible could suggest molecular mechanisms of YA function. Specifically, YA embryos arrest with condensed chromatin before the first mitosis (Lin et al. 1991), but it is not clear whether they arrest immediately prior to the first M-phase, after replicating their pronuclear DNA, or whether YA embryos arrest prior to the first embryonic S-phase, which normally occurs at the time of pronuclear apposition (Loppin, Bonnefoy, et al. 2005). Genetic epistasis data show that *Ya* functions upstream of *gnu, plu, and png*, as shown by the phenotype of the double mutants, which look like the *Ya* phenotype (Liu et al. 1997; Shamanski et al. 1991). Since the proteins encoded by these three genes make up the PAN GU kinase complex that couples S and M phases, and the multiple rounds of mitotic S phase characteristic of mutants in this complex do not occur in the absence of YA function, either YA is necessary for S phase, and *Ya* mutant embryos arrest prior to the first S phase, or YA is necessary for progression through the first cell cycle, so only one S phase can occur in *Ya* mutants or *Ya*;*gnu* double mutants. To distinguish between these possible models, I attempted to detect incorporation of BrdU in the male pronucleus of control and *Ya* mutant embryos. BrdU is a thymidine analog that is incorporated during DNA replication and can be detected using an antibody. Although it is possible to visualize the giant nuclei of *gnu* mutant embryos by this technique, I was unable to detect BrdU in pronuclei of early embryos.

Similarly, another group did not detect BrdU in prunuclei of activated, unfertilized eggs (Shamanski et al. 1991), although other evidence suggests that these nuclei do undergo one S phase (B. Loppin and C. Sunkel, personal communications).

D.2 Materials and Methods

Feeding: Oregon R P2 females (3-5 days old) were mated to ORP2 males overnight, and then the females were starved in empty vials with damp filter paper for 24 hrs. They were then put in vials where the only source of food was yeast and sugar made into a paste with 2mg/ml BrdU (Shamanski et al. 1991) solution. 0-30 minute collections of embryos were collected and fixed in Methanol/Heptane at 12, 24, 36, and 48 hrs after beginning BrdU feeding, and fresh BrdU-yeast paste was provided daily. In some experiments, virgin females were fed BrdU and then mated, followed by embryo collections.

BrdU incubation: After dechorionation in 50% bleach, 0-10 minute or 0-2hr collections of embryos were permeabilized in octane and incubated in 1mg/ml BrdU for 20 minutes (Sprenger et al. 1997).

BrdU detection: According to the method of P.H. O'Farrell (Sprenger et al. 1997), embryos in methanol were acid washed (2 X 15 min in 2.2N HCl with .01%Triton X-100), then neutralized (2 X 2 min 100mM borax), and washed 3 X 5 min PBST. Anti-BrdU primary antibody (Santa Cruz Bu20A) was diluted 1:20 in PBST with RNase (0.5-10 ug/ml) and incubated overnight with the embryos at 4 deg C. Anti-mouse Alexa-488 secondary (Invitrogen) was diluted 1:200-1:500, Fixed embryos were stained with propidium iodide and mounted in antifade as in (Horner et al. 2006).

D.3 Results and Discussion

Incubating embryos in BrdU solution provided 20min-2hr old embryos with BrdU incorporated in their DNA as a positive control for the BrdU detection method used. The youngest embryo from post-laying incubation that stained with anti-BrdU

had 8 nuclei (Fig D.1A), so the incubation method cannot be used to look at replication of apposed pronuclei. I tried in vitro activation in the presence of BrdU, and did not see any BrdU localized to DNA. This suggests that the vitelline membrane of mature oocyes is impermeable to BrdU. To expose developing oocytes to BrdU I fed starved females on BrdU-soaked sugar/yeast for 12hrs to four days as described in materials and methods. I never detected BrdU on the female meiotic products or other early cell cycle nuclei (Fig D.1B), although in some older embryos laid by BrdU-fed females I did see faint BrdU staining. Therefore there were difficulties both with detection and BrdU administration.

BrdU solutions needed to be prepared fresh (from frozen concentrated stock). To improve detection I tried two different secondary antibodies in addition to the one described in the materials and methods. They were TRITC and rhodamine secondaries, so I used DAPI as the DNA stain. Alexa 488 secondary antibody and propidium iodide DNA stain worked best. Instead of feeding yeast/sugar paste in an empty vial, I also tried making standard yeast/glucose media food with BrdU, but feeding in glass vials worked better.

Some conclusions made based on lack of BrdU staining in early Drosophila embryos are inconsistent with more recent data. Specifically, it was reported that meiotic products in unfertilized eggs do not replicate (Shamanski et al. 1991), but they do stain for PCNA (Chapter 5 and B. Loppin, personal communication). Because there is no BrdU staining on individual female meiotic products of wildtype eggs or embryos, I did not try to assay for DNA replication in Ya mutant eggs or embryos using BrdU labelling.

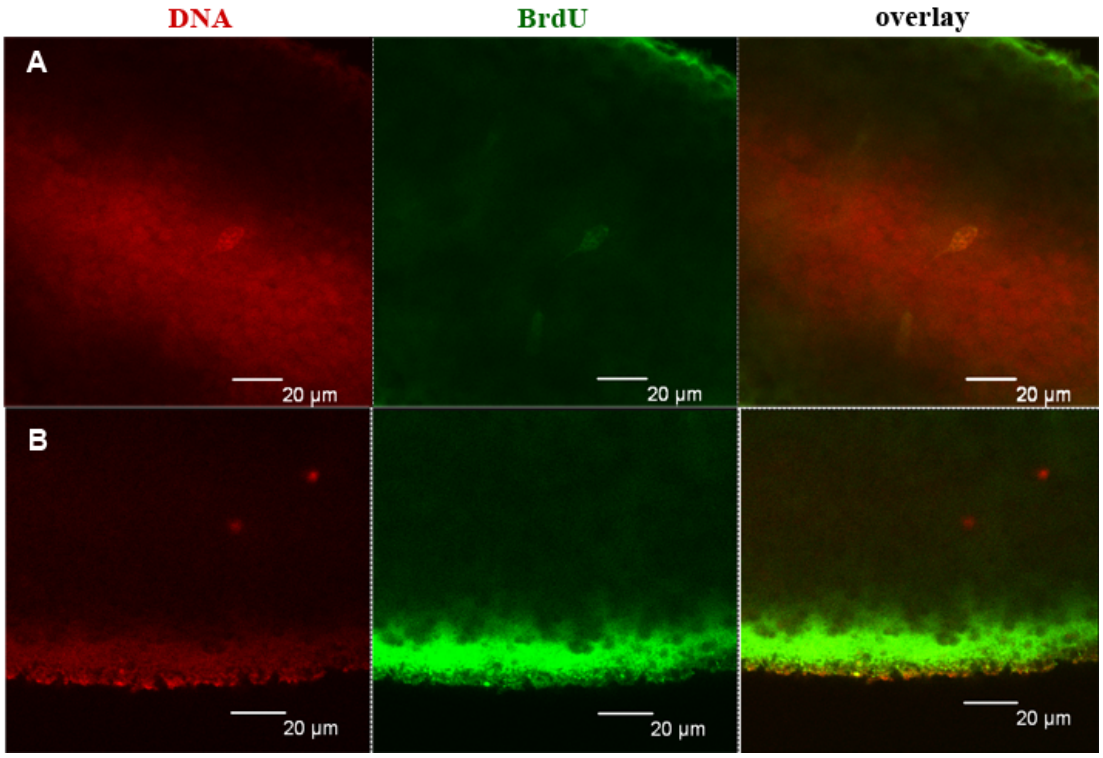


Figure D.1 BrdU incorporation was not detected in early embryos

DNA shown in red, BrdU shown in green. (A) BrdU-incubated embryo with eight nuclei (two visible in this plane) had BrdU staining. This embryo was the youngest observed from a 20-30 minute collection. (B) Embryo laid by BrdU-fed female does not stain for BrdU.

APPENDIX E

FLAG-YA INTERACTORS

E.1 Introduction

When purifying FLAG-YA for phospho-site identification (Chapter 3), I observed that more than one coomassie-stainable band was present in the eluate of the FLAG-YA purification. Because the additional bands could represent YA-interacting proteins that co-purify with YA, all the bands from a FLAG-YA purification from embryos were submitted for mass spectrometry to identify the proteins present.

E.2 Materials and Methods

FLAG-YA was purified from 0-1hr embryos exactly as in Chapter 3. The eluate was run on a 7.5%-20% gradient gel and stained with Simply Blue Safestain. Destained overnight in 3.3% NaCl solution. Cornell Proteomics Facility cut out the bands and performed tryptic digest and LC/MS/MS. A control purification of HIRA-FLAG produced much less total protein as observed by coomassie staining, because fewer embryos were used in the purification due to limitations on embryo collecting.

E.3 Results

The gel lanes with YA-FLAG and HIRA-FLAG eluates were divided into eight regions (Figure E.1) and proteins were identified by LC/MS/MS. Proteins present in both HIRA-FLAG and YA-FLAG samples were considered non-specific, such as Eukaryotic initiation factor 4B, an abundant component of the translation machinery. Proteins from intracellular compartments other than the nucleus (ER, miotchondria) were also eliminated from the list of possible YA-interactors, as well as abundant proteins such as actin and ribosomal proteins. I identified cell cycle regulators (especially components of the APC/C), nuclear proteins, kinases, and phosphatases as likely YA interactors, and they are listed in Table E.1.

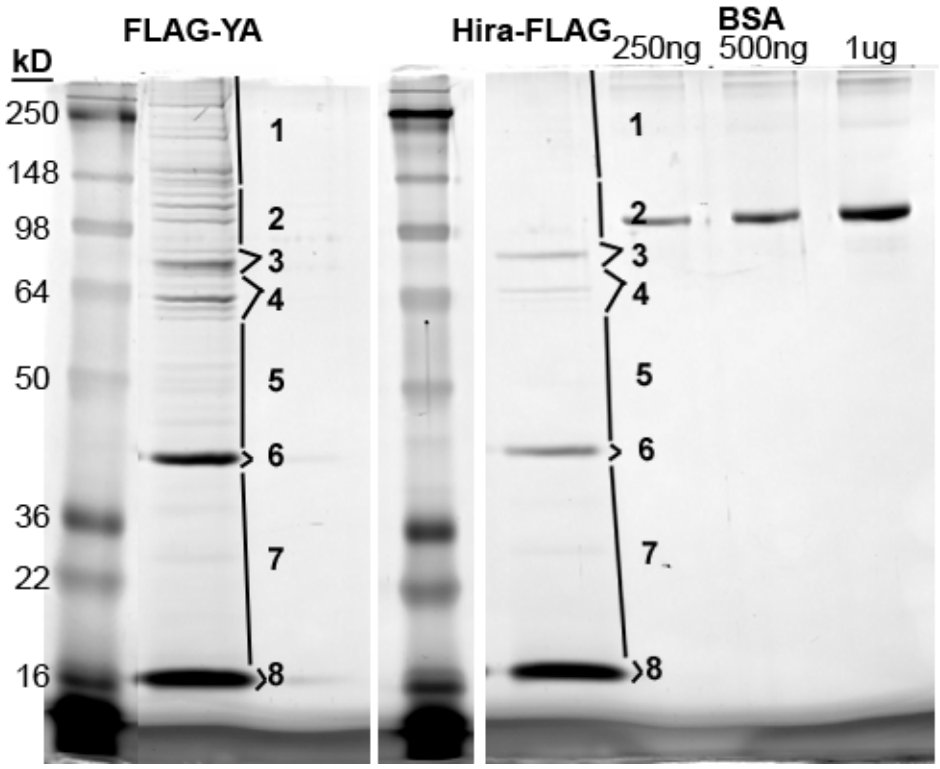


Figure E.1 Gel of purified proteins

SDS-PAGE gradient gel stained with coomassie (Simply Blue SafeStain) to visualize proteins. Darker staining of proteins from FLAG-YA purification (left) suggests more total protein was present than in HIRA-FLAG purification (middle). BSA standards (right) included for estimation of protein levels.

Table E.1 Proteins that co-purify with FLAG-YA but not HIRA-FLAG

Thirty-four proteins that co-purify with FLAG-YA.

band	prot_desc	prot_mass	prot_matches	annotation
1	shattered CG9198-PA	227128	50	APC1
1,2	dre4 CG1828-PA	127701	33, 8	FACT chromatin complex, replication
1, 2	cup CG11181-PA	127260	16, 3	
1,2, 3	Structure specific recognition protein CG4817-PA	81482	15, 11, 3	FACT chromatin complex, replication
1, 2, 3, 4	Cdc27 CG8610-PA	101220	13, 2, 7, 4	APC
1, 2	morula CG3060-PA	91535	6, 17	APC
1	CG15737 CG15737-PA	151218	5	wispy
1	Ack CG14992-PA	118344	4	protein tyrosine kinase, SH2-binding
1	Nup153 CG4453	196464	4	
1	Sir2 CG5216-PA	91779	4	chromatin
1	ubiquitin	8535	4	
1, 2	Anaphase Promoting Complex 4 CG32707-PA	87405	3, 12	APC
1	cdc23 CG2508-PA	77515	3	APC, Ub ligase
1	CG31687 CG31687-PA	80953	3	APC
1	multiple ankyrin repeats single KH domain CG33106-PA	422942	3	phos-Tyr signalling
1	smaug CG5263-PA	108997	2	
2	cdc23 CG2508-PA	77515	38	APC, Ub ligase
2	cdc16 CG6759-PA	81686	34	APC, Ub ligase
2	imaginal discs arrested CG10850-PA	88714	31	APC
2	Female sterile (2) Ketel CG2637-PA	98632	10	protein import into nucleus
3,4	pendulin (NLS-receptor), CG4799	57770	28,2	
3	Protein phosphatase 2A at 29B CG17291-PB	65382	2	
3	maternal expression at 31B CG4916-PA	51912	7	RNA helicase
3	Nucleosome assembly protein 1 CG5330-PA	42741	5	
4	FK506-binding protein 1 CG6226-PA	39319	5	nucleus, protein folding
5	Receptor of activated protein kinase C 1 CG7111-PA	35596	3	RACK
5	14-3-3epsilon CG31196	29780	2	
5	stubarista CG14792	35319	5	ribosome, mitotic spindle elongation
5, 6	Qm CG17521	26928	2, 8	ribosome, mitotic spindle elongation
6	CG6108 CG6108-PA	159450	9	nuclear gene
6	CG11419 CG11419-PA	22286	5	regulation of mitotic metaphase/anaphase transition, anaphase-promoting complex
6	Casein kinase II beta subunit CG15224	26067	2	
7	Histone H4 replacement CG3379-PC	11374	5	
8	CG6841 CG6841-PA	105162	6	nuclear gene

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