GENETIC AND MOLECULAR ANALYSIS OF CALCIUM SIGNALING PATHWAYS AND THE *PRAGE* GENE IN *DROSOPHILA* EGG ACTIVATION

A Thesis

Presented to the Faculty of Graduate School

of Cornell University

In Partial Fulfillment of the Requirements for the Degree of

Master of Science

by

Yun-Wei Lai

January 2011

ABSTRACT

Egg activation, the transition of mature oocytes into developing embryos, is initiated by different external signals in different animals. The signals include sperm entry, mechanical stimuli and pH changes. Although these signals are different, one response to those signals is well conserved: an increase of intracellular calcium (Ca^{2+}) level in the eggs. However, little is known about the molecular pathway downstream of the calcium rise that makes up egg activation.

Egg activation in Drosophila requires Ca^{2+} signaling. Horner (2006) and Takeo et al. (2006) showed that *sra*, a regulator of calcineurin (CN), has many defects in *Drosophila* egg activation. To dissect the role of CN further, I characterized the phenotypes of eggs laid by female expressing a constitutively-active CN (CnA^{act}) in *Drosophila* female germline. I have found that, both cell cycle resumption and MAPK dephosphoreylation are affected, although egg shell hardening and polyadenylation appears normal. This indicates that different events of egg activation may be controlled separately. Because CN is a major transducer of calcium signal that is known to be regulated by calmodulin (CaM), I chose to examine CaM and another major target of CaM that can be important for *Drosophila* egg activation: Ca^{2+}/Calmodulin-dependent protein kinase II (CamKII). Using transgenic expressed inhibitory peptides, I sought to inactivate functions of CaM and CamKII. However, I did not detect any effect on the hatchability of those inhibitory lines. There are several explanations. One possibility is that Ca^{2+} triggered egg activation events might not act through CaM-CamKII pathway or there are other regulators/molecular pathways that compensate for their functions. Alternatively, inhibitor peptides were expressed, but they may not have been present in sufficient amounts to inhibit their targets.

To understand the upstream regulator genes that control *Drosophila* activation,

I began characterizing *prage (prg)* gene. *Prg* mutant females lay eggs, but those eggs fail to destabilize maternal transcripts and never hatch (Tadros et al., 2003). These phenotypes indicate that *prg* may play a role in *Drosophila* egg activation. Thus, I examined egg activation phenotypes in oocytes and embryos from *prg* mutant females. I showed that VM cross-linking did not occur normally in embryos produced by both *prg* mutant alleles (prg^{16A} and prg^{32}). To find chromosome position of *prg* gene, Jun Cui previously conducted complementation analysis and DNA sequencing. His data suspected that CG14801, a possible exonuclease, is *prg* gene. In this thesis, I confirmed that CG14801 corresponds to *prg.* I assayed expression pattern of *prg* transcripts. The result shows that *prg* is expressed in adult of both sexes and the expression increases throughout embryogenesis.

In summary, my goal of this thesis is to identify the pathways through which calcium signal is transduced into egg activation in *Drosophila* and to determine the molecular targets and genes that involve in the pathway.

BIOGRAPHICAL SKETCH

Yun-Wei was born in Taipei, the capital city of Taiwan. She spent most of her childhood in the neighbor city, Taoyuan. Her family, including her younger sister Yun-Hsuan, always gave encouragements and supports for her to persuade her goals. As a child growing up in Tauyuan, Yun-Wei was fortunate to study in Futan High School, where she developed her interest in science and decided to study biology in college. In 2002, Yun-Wei got the admission from Animal Science Program of National Taiwan University (NTU) in Taipei. After the first year in college, Yun-Wei decided to transfer to Life Science Program since she found basic biology is more attractive to her. During her senior year, she joined Dr. Pung-Pung Hwang's lab in Academia Sinica as an undergraduate researcher, where she learned how to be a scientist. After she graduated from college, Yun-Wei felt she needed to learn something different. So she joined Master program in biochemistry at NTU. She studied developmental biology and molecular cell biology in lab, and learned the basic biochemistry in lecture. Two years of research seems not enough to her, Yun-Wei came to Cornell University where she continued her study in developmental biology in Wolfner's lab. After getting a Master degree, she will move to San Diego with her fiancé and seek a full time position in related field.

ACKNOWLEDGMENTS

I am deeply grateful to the support of many people during my time in Cornell. This thesis could not have been completed without their help. First, I would like to thanks my advisor, Mariana, for all the guidance and encouragement. She is a thoughtful mentor who advised me how to think profoundly about science. Her attitude and dedication to research inspired me a lot. I especially thank her for the input on my writing. I would also like to thanks my committee members, Kenneth Kemphues and John Schimenti, for the helpful instruction and suggestion on my research.

Special thanks to all members in the Wolfner lab. Everyone in the lab was so friendly and helpful. I especially acknowledge my colleagues on the “Egg activation” project, Jun Cui, Amber Krauchunas, Caroline Sartain and Norene Buehner for their help and suggestions on my projects. I want to thank all my friends in Cornell, Ella Chang, Chieh-Yang Cheng, Pi-chiang Hsu and Hojoong Kwak and many others. I appreciate to have all of you with me at Cornell, and thank for their treasure friendship.

I want to thank my parent for supportive and encouragement love. My entire live would never have been so wonderful without their effort. I appreciate to have my younger sister and also my best friend, Yun-Hsuan. Her kindly support and understanding helped me went through the most difficult time in my life. Finally, I want to thank my fiancé, Bernie Yang, with me. His love made me feel happy and satisfied. I love them with my whole heart.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1

INTRODUCTION

Mature oocytes differ in many ways from the cells that begin embryogenesis (Horner and Wolfner, 2008a; Parrington et al., 2007). The oocyte's outer coverings allow sperm binding and entry. Cell cycles of oocytes are meiotic, with specialized regulation to achieve proper chromosome segregation and production of haploid gametes (Jones, 2005). Meiosis is arrested at a specific stage, awaiting fertilization to modify all these features and to allow development to proceed. Also, many transcripts accumulated within the oocytes are not translated. Changes in the egg that allow it to transition to a developing embryo, upon fertilization, are known as egg activation (Horner and Wolfner, 2008b).

Egg activation is triggered by an external signal. This signal differs in different animals. In vertebrate and some marine invertebrates, activation is initiated by fertilization (Kurokawa et al., 2004; Runft et al., 2002). Insects including *Drosophila* do not use sperm to activate eggs; instead, an egg is activated during ovulation or when it passes through female reproductive tract (Heifetz et al., 2001; Horner and Wolfner, 2008b; Went and Krause, 1974). There are other ways to trigger activation, such as changes in pH value and ionic environment in some marine organisms (Lindsay et al., 1992; Santella et al., 1999).

Although the signals for egg activation differ, one downstream response is highly conserved: an increased intracellular calcium (Ca^{2+}) levels in the egg (Ducibella et al., 2006). The second section of this chapter focuses on the relationship between Ca^{2+} signaling and egg activation. Although how calcium triggers egg activation events

is not well understood, the molecular mechanisms of some steps of egg activation have been studied extensively. The first section of this chapter focuses on mechanisms of specific events of *Drosophila* egg activation.

1.1 Mechanism of egg activation

Modification of egg-coverings

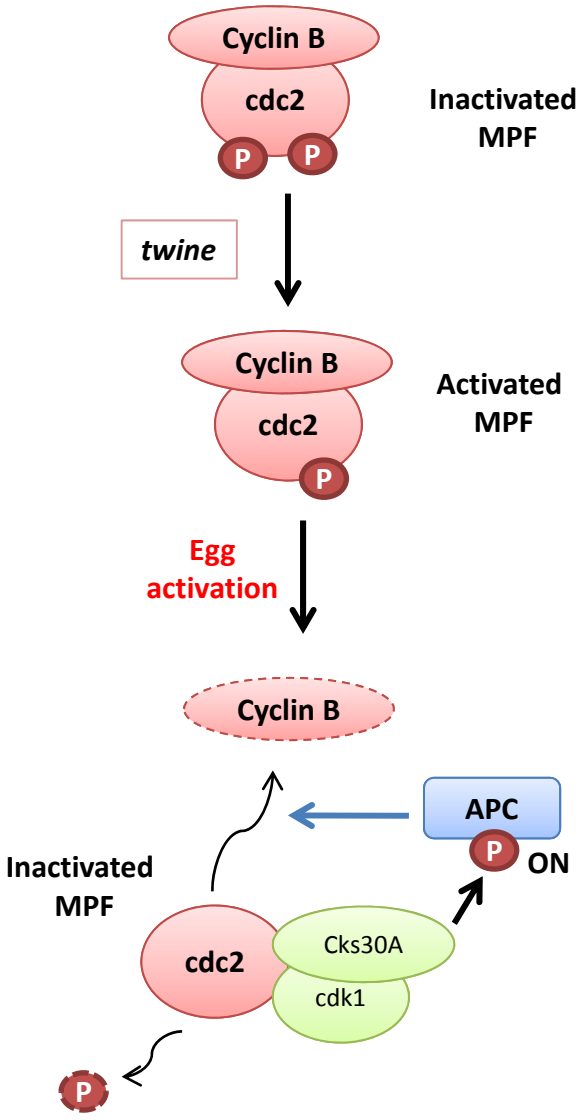
During activation, the outer coverings of the mature oocyte are modified to support and protect the future embryo and to prevent polyspermy. In many animals, these modifications occur by cortical granule (CG) exocytosis (Sun, 2003; Tsaadon et al., 2006; Wessel et al., 2001). Although *Drosophila* eggs do not contain CGs, the permeability of their vitelline envelopes changes during egg activation preventing diffusion-in of small molecules, presumably to protect embryos from external damage.

Drosophila oocytes are surrounded from outside to inside by a chorion, a waxy layer and a glycoprotein-rich vitelline membrane (VM) (Waring, 2000). The proteins of VM and chorion are cross-linked when eggs are activated, making impermeable barriers that block the entry of small molecules (Heifetz et al., 2001).

Release of oocyte meiotic arrest

The regulation of the cell cycles of oocytes during development is very important. One hallmark of this regulation is that meiosis arrests before egg activation (Jones, 2005). In many vertebrates, such as human, mouse and *Xenopus* (Fan and Sun, 2004), mature oocytes are arrested at metaphase II. In *Drosophila,* the cell cycle is arrested at metaphase I instead. To maintain the arrest in *Drosophila,* M-phase promoting factor (MPF) (cdk2-cyclinB) must be activated by *twine*, a homolog of Cdc25 (Figure 1.1) (Taylor et al., 2004; White-Cooper et al., 1993). Upon activation, Cks30A interacts with Cdk1 and binds to MPF, which promotes the phosphorylation of anaphase promoting complex (APC) (Swan et al., 2005). APC must be activated to allow the

Figure 1.1 Molecular mechanisms of meiosis arrest and release in *Drosophila.* Maturation-promoting factor (MPF) consists of a cyclin B protein and a cdc2 protein. *Twine* encodes the *Drosophila* homolog of Cdc25, a protein phosphatase that activates MPF. Activated MPF leads to meiotic arrest in *Drosophila* mature oocytes. Upon egg activation, the interaction of MPF and Cdk30A-cdk1 complex promotes phosphorylation of anaphase-promoting complex (APC), which further degrades and inactivates MPF.


Cyclin B
cdc2
P
P
Inactivated
MPF
twine
Cyclin B
cdc2
P
Activated
MPF
Egg
activation
Cyclin B
APC
P
ON
Inactivated
MPF
cdc2
Cks30A
cdk1
P

release of metaphase I arrest by MPF inactivation. Many molecules are also involved in regulating *Drosophila* cell cycle resumption, such as *cortex(cort)* and *fizzy(fzy)*, which encode members of the Cdc20 family (Page and Orr-Weaver, 1996; Swan et al., 2005; Swan and Schupbach, 2007). Mutants in those genes results in an earlier cell cycle arrest. For example, the majority of eggs from *cort* (or *fzy*) mutant females resumes meiosis, but re-arrest in metaphase of meiosis II. Also, embryos from double mutant *cort fzy* females arrest with elevated levels of mitotic drivers, including cyclin A, cyclin B and cyclin B3, which suggests that APC activity associated with *cort* and *fzy* is essential for *Drosophila* meiotic resumption (Liu and Maller, 2005; Rauh et al., 2005; Swan and Schupbach, 2007).

Changes in maternal mRNA populations

mRNAs and proteins are accumulated in oocytes during oogenesis. Upon activation, those maternal mRNA undergo post-transcriptional regulation to support embryonic development, until activation of the zygotic genome. Some maternal products are marked for degradation and others are targeted to be activated (Bettegowda and Smith, 2007; Tadros and Lipshitz, 2005). Maternal mRNAs whose products are necessary for early embryogenesis are activated for translation. For example, a poly (A) tail is added to the 3' end (3' UTR) of *bicoid (bcd)* mRNA during activation of *Drosophila* oocytes (Paillard and Osborne, 2003). This mechanism promotes translation of the *bcd* mRNA; similar phenomena occur for other maternal mRNAs, such as *Toll and torso* (Driever and Nusslein-Volhard, 1988). mRNAs for proteins that are no longer needed are degraded. Those transcripts contain degradation signals in the 3' UTRs, such as an A/U rich element (ARE) and embryo deadenylation element in *Xenopus*, targeting them for elimination (Bashirullah et al., 1999; Giraldez et al., 2006; Mishima et al., 2006). Coordinating these post-transcriptional modifications during oogenesis and oocyte maturation is important for supporting

early embryonic development.

Changes of protein phosphorylation states

Mitogen-activated protein kinases (MAPKs), which are normally active when phosphorylated, are used to integrate and transduce signals in many organisms, including yeasts, worms, flies and mammals (Brunet and Pouyssegur, 1997). MAPKs are involved in oocyte meiotic re-initiation, spindle assembly, metaphase II arrest during vertebrate oogenesis (Sun et al., 1999). Sackton et al. (2007) reported that MAPK activities change during *Drosophil*a egg activation: the enzymes are active (phosphorylated) in mature oocytes, and then are inactivated (dephosphorylated) during egg activation (Sackton et al., 2007). Although it is not yet clear how the MAPK pathway is regulated to effect egg activation, it is possible that MAPKs respond to Ca^{2+} signals and either directly or indirectly mediate meiotic cell cycle arrest (Nebreda and Hunt, 1993).

1.2 Calcium signaling and egg activation

Increases in the intracellular level of free Ca^{2+} during egg activation are observed in many animal species (Ducibella et al., 2006; Stricker, 1999). This common event can ensure the initiation and progression of normal development (Berridge et al., 2003; Lee et al., 2006). For example, in mammals, the fertilizing sperm triggers oscillatory Ca^{2+} waves that trigger egg activation events, including CGs exocytosis, pronuclear (PN) formation, cell cycle release and secondary polar body extrusion (Ducibella et al., 2002; Halet et al., 2003). Those findings raise the importance of exploring what signals trigger Ca^{2+} changes (upstream) and how Ca^{2+} leads to egg activation events (downstream).

The signaling upstream of the Ca^{2+} oscillation is often regulated by 1, 4, 5-inositol triphosphate (IP_3) in mammals (Miyazaki et al., 1993). IP_3 is a secondary messenger molecule produced when phospholipase C (PLC) hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP_2). Upon fertilization, IP_3 levels increase, along with the Ca^{2+} rise. When agonists of IP_3 are injected into mammalian eggs, Ca^{2+} is released and oscillations are increased (Miyazaki, 1988). The mammalian sperm-specific PLCζ causes intracellular Ca^{2+} release by catalyzing the production of IP_3 (Figure 1.2). Ca^{2+} is released through the IP_3 receptor, IP_3R-1, which is located in the membrane of the endoplasmic reticulum (ER) (Krizanova and Ondrias, 2003; Swann et al., 2006). This Ca^{2+} oscillation triggers mammalian egg activation events, including resumption and completion of meiosis, blockage of polyspermy and translation of maternal mRNAs (Ajduk et al., 2008).

Ca^{2+} in oocytes and eggs regulates downstream proteins through a variety of signaling pathways. In mouse and *Xenopus*, a Ca^{2+}/Calmodulin-dependent protein kinase II (CamKII) has been reported to be necessary for activating several downstream events, such as modifying the vitelline membrane (VM) to block polyspermy and changes in mRNA and protein populations (Ducibella et al., 2002; Markoulaki et al., 2004; Schultz and Kopf, 1995; Tatone et al., 2002). A working model is presented that integrates evidence to suggest a relationship of upstream Ca^{2+} to the protein kinase (CamKII) and the downstream egg activation events in mammals (Figure 1.3) (Ducibella and Fissore, 2008). In brief, increased Ca^{2+} binds CaM, which activates CamKII. CamKII mediates down-regulation of maturation M-phase promotion factor (MPF), releasing cell cycle arrest. In *Xenopus*, the modulation of the phosphatase calcineurin (CN) activity has also been shown to be essential for processing of egg activation (Mochida and Hunt, 2007; Nishiyama et al., 2007). The

Figure 1.2 Ca^{2+} signaling pathway during mammalian fertilization. The binding and fusion of a sperm with oocytes introduces PLCζ into the egg's cytoplasm. PLCζ hydrolyzes PIP_2 and produces $InsP_3$ (IP_3). IP_3 binds to IP_3 receptor, IP_3R, on endoplasmic reticulum (ER) to release internal stored Ca^{2+}. Ca^{2+} activates the downstream pathways by binding to calmodulin (CaM) (green pathway). In echinoderms, NADDP activates membrane Ca^{2+} channels which allow the entry of external Ca^{2+} (red pathway). Figure modified from Whitaker (2006).

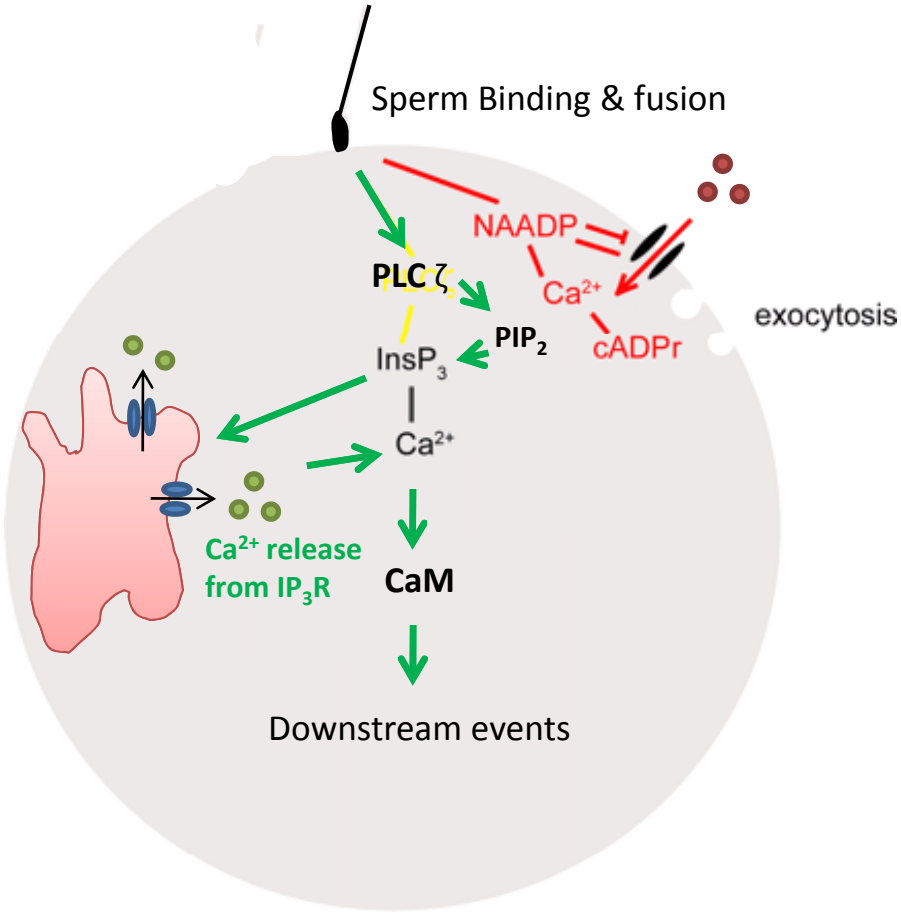
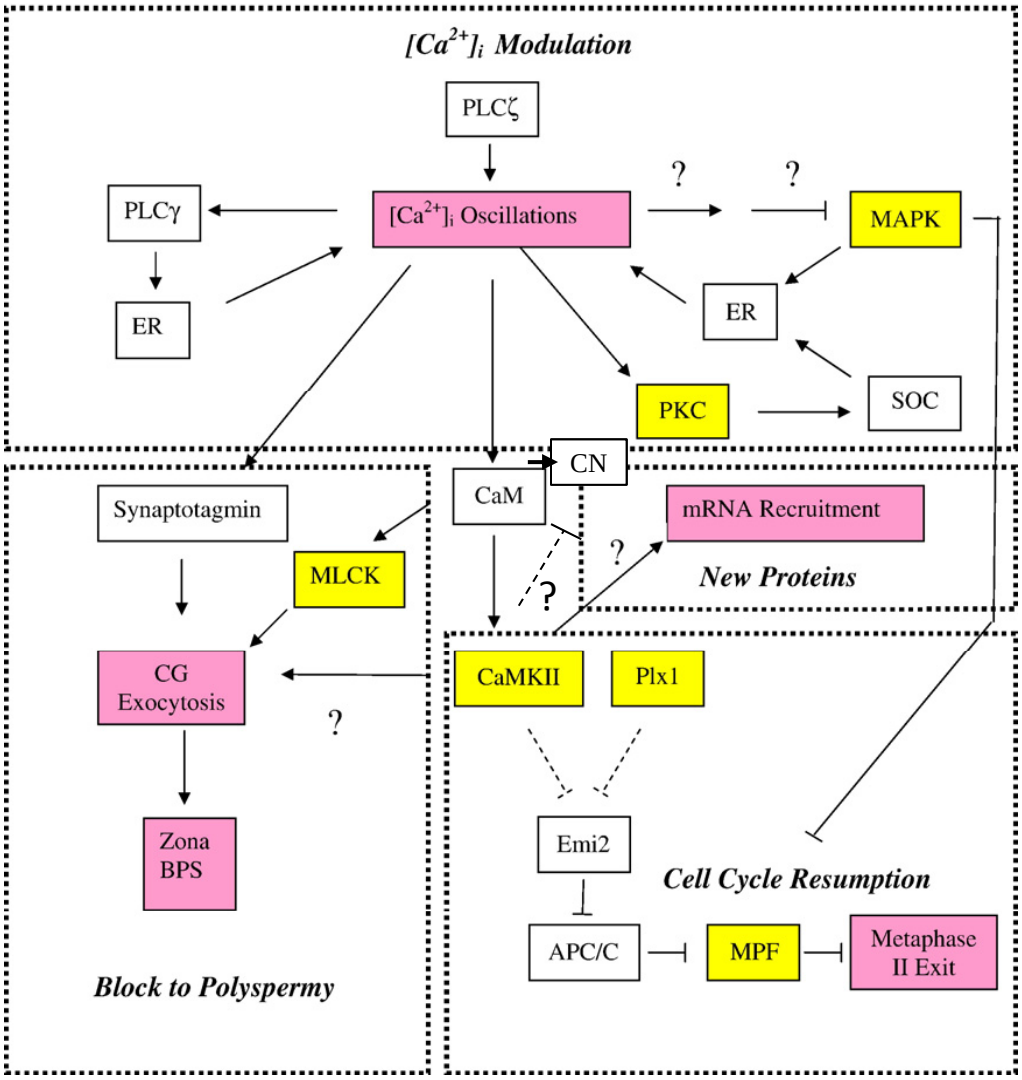

Sperm Binding & fusion
NAADP
PLC ζ
Ca2+
exocytosis
cADPr
PIP2
InsP3
Ca2+
Ca2+ release
from IP3R
CaM
Downstream events

Figure 1.3 Relationship between upstream Ca^{2+} signals and downstream egg activation events in mammal. This is a simplified version of the Ca^{2+} signaling model presented by Ducibella and Fissore (2008). These Ca^{2+}-dependent mechanisms regulate the activities of protein kinases/phosphatases, which promote downstream events of egg activation. For example, CamKII regulates cell cycle resumption through the inhibition of MPF. CG exocytosis is modulated by MLCK, CamkII and synaptotagmin. The detailed relationships and interactions between those molecules/pathways are still unknown. Abbreviations: CaM: calmodulin, CamKII: Ca^{2+}/CaM-dependent protein kinase II, CN: calcineurin. PLC: phospholipase C, ER: endoplasmic reticulum, MAPK, MAP kinase, PKC: protein kinase C, SOC: store-operated channels. MLCK: myosin light chain kinase, CG: cortical granule, BPS: block to polyspermy. Emi2, early mitotic inhibitor 2, APC/C: cyclosome, MPF: maturation promoting factor. Figure modified from Ducibella and Fissore (2008).


[Ca2+]i Modulation
PLCζ
PLCγ
[Ca2+]i Oscillations
?
?
MAPK
ER
ER
SOC
PKC
CN
CaM
Synaptotagmin
MLCK
mRNA Recruitment
New Proteins
?
?
CG
Exocytosis
?
CaMKII
Plx1
Zona
BPS
Emi2
Cell Cycle Resumption
APC/C
MPF
Metaphase
II Exit
Block to Polyspermy

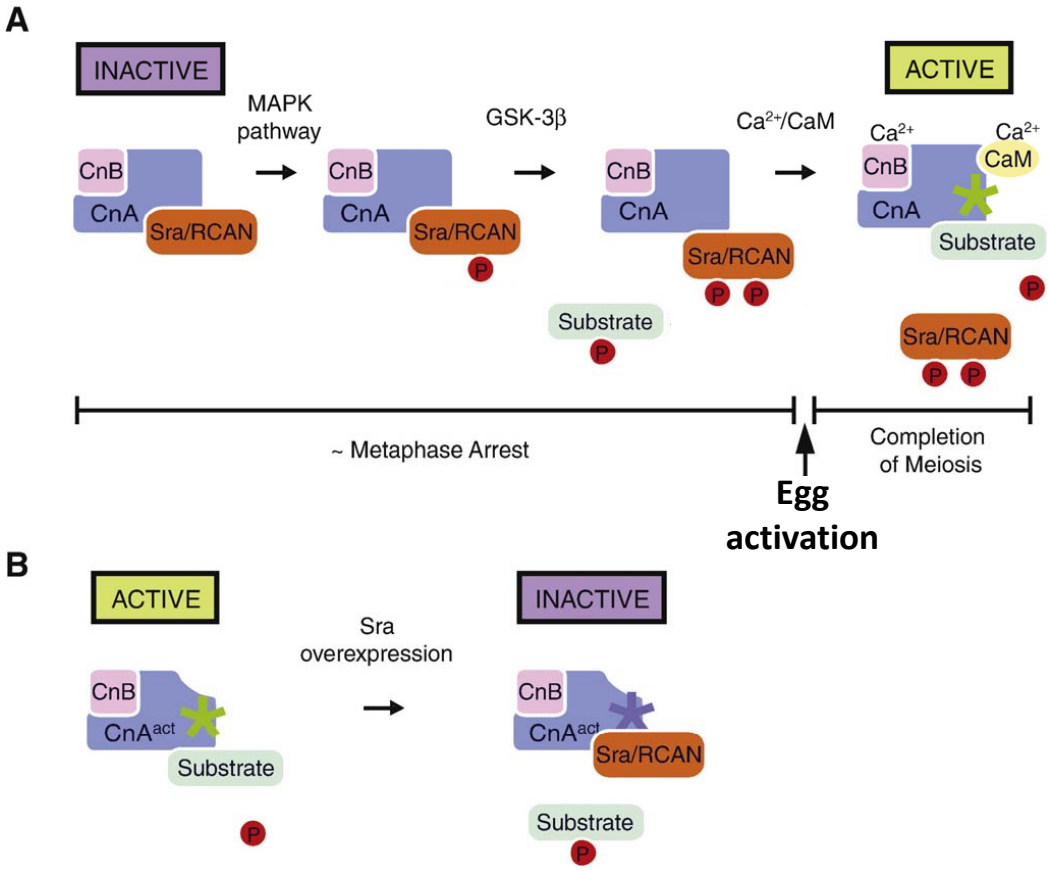
CN level, which changes independent of CamKII, is transiently increased immediately after the Ca^{2+} addition to *Xenopus* egg extracts.

Though those studies indicated the role of Ca^{2+}/CaM regulated protein kinase and phosphatase in modulating egg activation events in mammals, it is not clear that if the Ca^{2+} signal is transduced only through CamKII and CN pathways or coordinates with other pathways to regulate activation. Also, the Ca^{2+}-dependent signaling mechanisms that cause egg activation events in *Drosophila* are yet to be fully understood.

Mechanical stimulation activates *Drosophila* oocytes *in vitro.* In addition, exogenous calcium is essential for *Drosophila* egg activation *in vitro* (Horner and Wolfner, 2008a). Mature *Drosophila* oocytes cannot be fully activated in the absence of Ca^{2+} or when treated with the Ca^{2+} chelator BAPTA (1,2-Bis(2-aminophenoxy) ethane-N,N,N′,N′-tetraacetic acid tetrapotassium salt). *Drosophila* mutants in the *sarah (sra)* gene, which encodes calcipressin, a regulator of CN, lay eggs defective in many aspects of egg activation (Horner et al., 2006; Takeo et al., 2006). CN regulated cell cycle completion of *Drosophila* has been shown to be stimulated by the phosphorylation of *sra* (Takeo et al., 2010). Before activation, CN is kept inactive by the inhibitory binding of SRA (Figure 1.4 A). During *Drosophila* female metaphase arrest, SRA is sequentially phosphorylated by MAPK and GSK-3β, respectively. Phosphorylated SRA is then released from CN, once the eggs are activated. CN activation is triggered by binding of Ca^{2+}/CaM, which dephosphorylates unknown substrates to allow the egg to resume meiosis and maybe also catalyze other activation events. Figure 1.4 B summarize a model for how *sra* overexpression suppresses CN.

Given that *sra* only partially affects activation processes in *Drosophila*, I think the calcium signaling pathways, other than only the involving CN, may also be

Figure 1.4 Model of calcineurin regulation by SRA/RCN. (A) During *Drosophila* female meiotic arrest, calcineurin (CN) is inactivated by *sra* binding. Two serine residues of *sra* were sequentially phosphorylated by MAPK and GSK-3β pathways, respectively. These phosphorylations are essential to release *sra*. Upon activation, CN is activated by Ca^{2+}/CaM binding, which dephosphrylate unknown substrate(s) to allow the cell cycle resumption. (B) overexpression of *sra* inhibits active CN. This provides a model of how *sra* overexpression suppresses CnA^{act} phenotypes. Figure adapted from Takeo et al. (2010)


A
INACTIVE
MAPK pathway
GSK-3β
Ca2+/CaM
ACTIVE
Ca2+
Ca2+
CnB
CnA
Sra/RCAN
CaM
Substrate
P
~ Metaphase Arrest
Completion of Meiosis
Egg activation
B
ACTIVE
Sra overexpression
INACTIVE
CnA act

involved in *Drosophila* egg activation. Very little is known about the involvements and interactions of Ca^{2+}-dependent molecules in *Drosophila* egg activation, so far only CN has been identified to be important. But studies in other organisms, such as mouse and *Xenopus*, focus on another Ca^{2+}-depent protein kinase: CamKII. Both CN and CamKII are highly conserved Ca^{2+} effectors and are strictly regulated by CaM. However, there is no direct evidence in *Drosophila* to suggest that CaM and CamKII are involved in egg activation. It is also not clear if there is any relationship between these molecules in *Drosophila* egg activation. In this thesis, I examine the roles of all three Ca^{2+} signal players, CaM, CN and CamKII, in *Drosophila* egg activation (Chapter 2).

1.3 The female-sterile mutant: *prage (prg)*

Several *Drosophila* genes have been identified as regulating egg activation. These genes include *cortex* (*cort*), *grauzone* (*grau*) and *sarah* (*sra*) and *wispy (wisp)* (Cui et al., 2008; Horner et al., 2006; Page and Orr-Weaver, 1996; Tadros et al., 2003; Takeo et al., 2006). Eggs laid by females mutant in any of these genes proceed to normal (or relatively normal; *wisp*) mature oocyte stage before activation, but are defective in egg activation events later on. *Cort* encodes a *Drosophila* CDC20 which up-regulates the activity of anaphase-promoting complex/cyclosome (APC/C) and releases the meiotic cell cycle arrest upon activation (Chu et al., 2001; Pesin and Orr-Weaver, 2007; Swan and Schupbach, 2007). *Grau* encodes a transcription factor required for *co*rt expression. Mutations in *cort,* like mutants in *grau*, cause abnormal metaphase arrest in meiosis II (Chen et al., 2000; Harms et al., 2000; Lieberfarb et al., 1996; Page and Orr-Weaver, 1996; Swan and Schupbach, 2007). As noted above, *sra* encodes calcipressin, which is a regulator of the calcium-dependent phosphotase calcineurin. Eggs from *sra* mutants are defective in many aspects in egg activation (Horner et al., 2006; Takeo et al., 2010;

Takeo et al., 2006). As described above, maternal mRNAs and proteins that are deposited into the developing oocyte regulate early development. Some of these maternal transcripts are activated by poly(A) addition; some are degraded during early embryogenesis. The eggs laid by *wisp* mutant mothers fail to destabilize those maternal transcripts (Tadros et al., 2003). *Wisp* encodes a cytoplasmic poly(A) polymerase (PAP) in the GLD-2 family, which regulates poly(A) tail length in eggs. Mutants in *wisp* result in eggs with several egg activation defects, including early cell cycle arrest and failure of *bicoid* mRNA polyadenylation and translation (Benoit et al., 2005; Brent et al., 2000; Cui et al., 2008). WISP is needed for poly(A) tail elongation of maternal mRNAs, including *bicoid*, *Toll* and *torso*. However, it is not yet known how *wisp* activity is coordinated in terms of regulation by Ca^{2+} or regulation downstream activation events.

Prg is a female-sterile mutant that was identified in the same screen as *wisp*. Eggs from *prg* mutant females have defects in mRNA translation, degradation and cell cycle completion, but the exact timing of their cell cycle arrest is still unknown. *Prg* was first mapped to 1B4-1E2, a region that contains around 180 predicted genes (Tadros et al., 2003). Jun Cui further narrowed down the *prg* region to 1B10-13 by complementation testing with deficiencies from the Exelixis collection (Artavanis-Tsakonas, 2004; Bonds et al., 2007; Parks et al., 2004). The endpoints of the deficiencies localized *prg* to a region containing 15 genes**.** Jun further found that only one deficiency, *Df(1)BSC719/*Binsinscy, failed to complement both prg^{16A} and prg^{32} mutant alleles. This narrowed the list of candidate genes for *prg* to only 10 genes. Jun sequenced both *prg* mutants in these 10 genes, and found that both prg^{16A} and prg^{32} have molecular lesions in CG14801, a predicated exonuclease, suggesting that this could be the *prg* gene. In Chapter 3, I further describe complementation analysis with

P-element insertion lines from the Bloomington Stock Center that confirmed the CG14801 as *prg*. Also, I report characterization of additional phenotypes of embryos of *prg* mutant females.

1.4 Conclusion

Egg activation is a complex and delicate process that enables the embryo to develop properly. It involves many pathways and molecules. Studies have focused on the Ca^{2+} signals that trigger activation and on kinases that are necessary for subsequent events (Figure 1.3). However, the link between these upstream signals and the downstream molecules that carry out activation is still unknown. My elucidation of the players and mechanisms in egg activation can be important in understanding the basis of their developmental transition.

1.5 Thesis outline

The purpose of present study was to identify the regulators/effectors that modulate egg activation and to explore the molecular activation pathway in *Drosophila melanogaster*. Part of my research concerned the *sra*/CN pathway. In Chapter 2, I examine three major downstream Ca^{2+} molecules that can be important for regulating *Drosophila* egg activation events. I first tested the effects of constitutively-active CN A subunit (CnA^{act}) on *Drosophila* egg activation. I used the standard assays for testing egg shell hardening, meiosis resumption, *bicoid* polyadenylation and MAPK dephosphorylation (Cui et al., 2008; Horner et al., 2006). My results show that CN acts in some but not all activation events, suggesting the pathways that control those events

may be divergent, and that same effect differs in *sra* mutant versus CnA^{act}. For example, *sra* mutant affects cell cycle and polyadenylation, while CnA^{act} mutant has defects in cell cycle and MAPK regulation. My results confirm the involvement of CN in *Drosophila* egg activation.

Because calmodulin (CaM) is the major modulator of CN, it is probably the upstream regulator of CN in modulating *Drosophila* egg activation (Horner et al., 2006; Takeo et al., 2006). I attempted to inhibit CaM and another CaM target, CamKII, using transgenic expression inhibitor peptides, but did not see abnormalities in egg activation. Possible explanations for this negative result will be discussed in the same Chapter.

In Chapter 3, I began to characterize the *prg* gene, which is necessary for egg activation. I used complementation tests to confirm that CG14801 as *prg* gene. To assay expression pattern of *prg*, I performed RT-PCR for *prg* mRNA. Transcripts of *prg* were detected in both male and female adult flies, and expression increases throughout embyogenesis. I further characterized the *prg* effects in egg activation. Eggs lacking PRG function are abnormal in VM protein cross-linking, since most such embryos do not become bleach resistant. Further examinations are in the process to help dissect the mechanism that control egg activation.

CHAPTER 2

CALCIUM SIGNALING PATHWAY IN *DROSOPHILA* EGG ACTIVATION

2.1 Introduction

Egg activation is a critical cellular event required to initiate development of a fertilized egg. It leads to the transition of fertilized mature oocytes into developing embryos. It is initiated by external signals. These signals are different in different animals. In most animals, such as vertebrates and most marine invertebrates, the fertilizing sperm triggers egg activation. In insects, however, egg activation is triggered by passage of the egg through the female reproductive tract (Heifetz et al., 2001). Despite the diversity in initial triggers for activation, one response to those signals is well conserved: an increase of intracellular free calcium (Ca^{2+}) levels in the egg. These changes in Ca^{2+} concentration are both necessary and sufficient for at least two egg activation events in mammalian eggs: cell cycle completion and cortical granule release (Chang et al., 2009). However, little is known about the molecular pathway downstream of the calcium rise that signals egg activation.

Calmodulin (CaM), the calcium binding protein, plays a pivotal role in regulating cellular response of Ca^{2+} signaling in all eukaryotic cells (Andruss et al., 2004). Increased Ca^{2+} levels in cells enhance the binding of Ca^{2+} to CaM, resulting in conformational changes of CaM. The activated CaM then triggers its downstream signal transducers. In mammals and *Xenopus,* two major CaM targets that have been reported to regulate parts of egg activation events are Ca^{2+}/CaM-dependent protein kinase II (CaMKII) and calcineurin (CN) (Chang et al., 2009; Takeo et al., 2010).

Calcineurin (CN) is a highly conserved phosphatase that responds strictly to the Ca^{2+} signal and CaM activity (Rusnak and Mertz, 2000). It is composed of two

subunits: the catalytic A subunit (CnA) and the regulatory B subunit (CnB). In mammals and yeast, regulators of calcineurin (RCNs) bind to CnA, and act as CN inhibitors when they were overexpressed (Fuentes et al., 2000; Kingsbury and Cunningham, 2000; Sanna et al., 2006). However, some studies suggested that RCN can be both activator and inhibitor of CN: it facilitates CN at low concentration, while it inhibits CN when it is overexpressed (Hilioti et al., 2004; Mehta et al., 2009). Previous study showed that egg activation in *Drosophila* requires calcipressin, an RCN (Horner et al., 2006; Takeo et al., 2006). Eggs of *sarah (sra)* mutant females, which lack calcipressin, are defective in many aspects of egg activation. Takeo et al. (2010) demonstrated that *sra* is required to positively regulate CN and promotes female meiosis in *Drosophila*, since the phenotypes of dominant-negative CnA mutants (CnA^{DN}) are enhanced when *sra* activity was reduced (Takeo et al., 2010). Although how RCNs regulate CN in terms of egg activation is unclear, these results indicate the importance of the role of CN and its regulators in triggering downstream pathways of egg activaion. Given that CN is essential for egg activation in *Drosophila* (Horner et al., 2006; Takeo et al., 2006), we hypothesize that CaM-CN pathway could also be important.

Another major target, and transducer, of calcium regulation is CamKII. Previous studies also uncovered a relationship between cell cycle resumption and CamKII regulation in vertebrates (Lorca et al., 1993). Using *Xenopus* egg extracts, Lorca et al. found that the inactivation of MPF and destruction of cyclin protein were controlled by CamKII activity. Constitutively-active CamKII mouse eggs triggered several egg activation events even in the absence of an elevated Ca^{2+} level (Knott et al., 2006). CamKII is also required for CG exocytosis and polyadenlyation in mouse, since the inhibition of CamKII blocks these processes (Atkins et al., 2004; Tatone et al., 1999). Whether *Drosophila* CamKII has similar importance in egg activation is

unknown. Therefore, the CaM-CamKII pathway is another candidate for studying egg activation in *Drosophila.* Standard genetic assays cannot be used to examine the roles of CaM and CamKII in *Drososphila* egg activation, as will be presented in the Results and Discussion in this Chapter.

To continue dissecting the role of CN in triggering the egg activation, I have characterized phenotypes of eggs laid by females expressing CnA^{act} in the germline. As noted above, *Drosophila* CN is composed of two subunits, CnA and CnB. In *Drosophila,* three genes encode CnA (*CanA1*, *Pp2B-14D* and *Can-14F*) and two genes encode CnB (*CanB* and *CanB2*). Takeo et al. (2006) showed that only *Pp2B-14D*, *Can-14F* and *CanB2* are expressed in early embryos and ovaries, and presumably function in female germline (Takeo et al., 2006). CnA^{act} was created from *Pp2B-14D* by inserting a stop codon at C terminal part of the gene, which results in an active form of CnA (Takeo et al., 2006). I report here that many molecular aspects of egg activation events are blocked in CnA^{act} eggs, although membrane cross-linking and polyadenylation of maternal mRNAs occur normally. This indicates that CN is involved in *Drosophila* egg activation and different events of egg activation may be controlled independently. To determine the role of CaM and CamKII, I assessed the female hatchability by using inhibitors to block their functions in eggs. I was unable to detect effects of expression of these inhibitors in female hatchability; possible explanations for my results will be discussed.

2.2 Materials and Methods

Fly stocks: All *Drosophila melanogaster* stocks were raised on yeast-glucose-agar medium at room temperature (23±2°C) in a 12 hr light: 12 hr dark day circle. Either the P2 strain of Oregon R or w^{1118} was used as the wild-type stock. A stock yielding CnA^{act} transgenic flies, *nanos*-GAL4 (*nos*-GAL4), *UASp-Pp2B-14D*^{act} /TM3,

was kindly provided by Satomi Takeo and Toshiro Aigaki, Tokyo Metropolitan University, Japan. Transgene expression was driven with either *nos*-GAL4 (Van Doren et al., 1998) or *maternal tubulin*-GAL4 (*mat*-GAL4; line 7062 from Bloomington Stock Center) (Hacker et al., 1997). In addition, our lab previously generated transgenic lines to express inhibitor peptides for CaM or CamKII: 1) "KA" inhibitor peptide sequence for CaM (VanBerkum and Goodman, 1995): QKDTKNMEAKKLSKDRMKKYMARRKWQKTGHAVRAIGRLSS. 2) "Ala" inhibitor peptide sequence for CamKII (Griffith et al., 1993): RSTVASMHRQEAVDCLKKFNARRKLKGA. Our lab moved sequences encoding each peptide into the germline expression vector pUASP (Rorth, 1998) and generated stable homozygous transgenic lines for these. To drive peptides in oogenesis, I crossed these flies either to *nos*-GAL4 or *mat*-GAL4 and hatchability of the eggs laid by those progeny was examined.

In vitro *egg activation:* The protocol of *in vitro* egg activation is modified from Page and Orr-Weaver (1997). Stage 14 mature oocytes were dissected from ovaries of 3-5 days old virgin females in hypertonic Isolation Buffer (IB); eggs in IB remain inactivated. The dissected oocytes were then incubated in hypotonic Activation Buffer (AB) for 25 min to activate the eggs (AB was removed and replaced with fresh AB every 5 min). Activated oocytes were then selected in 50% bleach (Page and Orr-Weaver, 1997).

Bleach assay: Bleach assay is also called Vitelline membrane hardening assay (Horner et al., 2006). Basically, the proteins within vitelline membrane become cross-linked in activated eggs. This change makes eggs resistant to lysis by bleach since the small $HClO_3$ molecules cannot get through the VMs. In contrast, the VMs of unactivated oocytes are cross-linked and thus can be penetrated by bleach. Unactivated eggs incubated in 50% bleach lyse within 2 min. Embryos from females 3-5 days after

mating with wild-type Oregon R P2 males were collected on grape juice-agar media. The embryos then washed off from media plates with egg wash buffer (0.4% NaCl, 0.2 % Triton X-100). The collected embryos were placed into 50% bleach for 2 min and rinsed with water. The number of bleach resistant and lysed eggs was recorded.

Immunostaining of ovaries and embryos: Laid eggs were obtained as previously described (Horner et al., 2006). The dechorionated embryos were fixed in methanol/heptane. Fixed embryos were stored in 4°C in methanol. Fixed eggs were rehydrated in 1XPBST (1XPBS with 0.1% Triton X-100) with 5 min wash each for three times, prior to immunostaining. Ovaries from aged females were dissected and fixed in 4% fresh paraformaldehyde. A monoclonal mouse anti-α-tubulin (Sigma, St Louis, MO) antibody containing 5µg/ml RNAase (Roche Applied Science, Indianapolis, IN) was used at a 1:400 dilution in PBST, ovaries or embryos were incubated with this antibody, at 4°C overnight. The eggs were washed in PBST three times for 5 min each the next day. Then secondary antibody, Alexa Fluor 488-conjugated goat anti-mouse antibody (Invitrogen, Carlsbad, CA, USA) was added, at a dilution of 1:200 in PBST, for 2 hr in dark at room temperature. Eggs were washed in PBST five times. During the last wash, propidium iodide (Invitrogen) at a concentration of 10µg/ml was added to stain the DNA. Stained eggs were mounted on glass microscope slides in 75% glycerol containing 940 mM *n*-propyl gallate. Samples were analyzed using confocal microscopy [Leica TCS SP2 system equipped with an argon-krypton laser and coupled to a Leica DMRBE microscope (Leica Microsystems, Germany)].

Western blot analysis: Samples were collected for western blotting and prepared as described in (Horner et al., 2006). Samples were lysed in 20 µl protease and phosphatase inhibiting homogenization buffer and 20 µl of sample buffer was then added. Samples were run on 10.6% polyacrylamide gels. The proteins we transferred

to memberanes for western blot and incubated with anti-pERK (1:1,000), anti-p38 (1:500), anti-pJNK (1:250), anti-total ERK (1:1,000) and anti-tubulin (1:10,000) (Sigma) according to procedures in Sackton et al. (2007). Blots were incubated with Secondary antibody, and processed for visualization with either ECL or ECL+. Signal intensity was quantified and adjusted using image J (http://rsbweb.nih.gov/ij/).

Poly (A) tail assay: Oocytes from 20 aged virgin females (control and CnA^{act} expressing) were dissected and collected for each sample. 500-1000 embryos for the desired period of time were collected as described above. Total RNA was extracted with TRIzol (Invitrogen). PCR-based PAT assays were conducted as described in (Cui et al., 2008). Poly(A) tails of mRNAs were saturated by incubating in the cocktail with 20 ng 5'-phosphorylated oligonucleotide $p(dT)_{18}$. The $p(dT)_{18}$ was 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. The poly(A) tails were then ligated with poly(T) when adding 200 ng oligo$(dT)_{12}$-anchor. The mRNAs were reversed transcribed to synthesize PAT cDNA. PCR was used to detect the length of poly(A) tail of the mRNA. Sequence of primers for *bicoid* and oligo$(dT)_{12}$-anchor were described as (Salles and Stricker, 1995). PCR products were run on 1.5 % agarose gel.

Hatchability assay: Virgin females of inhibitor peptide (IP) (*nos-/ mat-* GAL4; UASP-IP), and w^{1118} males were collected. For a hatchability assays, 3-5 day-old females were mated with desired males in a vial. The male was removed after mating, the female was allowed to lay eggs for 24 hr. Females were transferred into new vials every 24 hr for 5-10 days. The number of eggs and the number of progeny was scored.

RNA preparation and RT-PCR: Samples were collected as described above, and homogenized in 1 ml Trizol Reagent (Invitrogen). 0.25 ml choloroform was added to the samples, which were then shaken vigorously, and incubated for 3 min at room temperature. After centrifugation for 15 min at 12,000 rpm, 4 °C, supernatants were

mixed with equal volumes of isopropanol, and then incubated at room temperature for 15 min. RNA was precipitated by another 12,000 rpm centrifugation at 4 °C for 20 min, washed with 75% ethanol and stored at -20 °C before use. The total RNA was treated with 1 µl DNAase for 30 min at 37 °C (Promega, Madison, WI, USA) to remove genomic DNA. The amount and quality of the total RNA were determined by measuring the absorbance at 260 nm and 280 nm with a NanoDrop 2000c spectrophotometer (Thermo, Waltham, MA, USA).

For cDNA synthesis, 5 µg of total RNA was reverse transcribed in a final volume of 20 µl containing 0.5 mM dNTPs, 2.5 µM oligo $(dT)_{18}$, 5 mM dithiothreitol, and 200 units superscript reverse transcriptase III (Invitrogen) for 50 min at 42 °C and followed an 15 min incubation at 70 °C. For PCR amplification, 1 µl cDNA was used as template in a 25 µL final reaction volume containing 0.25 mM dNTP, 1.25 units Gen-Taq polymerase (Genemark, Taipei, Taiwan), and 0.2 µM of each primer. The primer sets for the PCR of inhibitor peptides were:

UASp forward: 5'- GGCAAGGGTCGAGTCGATAG -3'

K10 reverse: 5'- TGGTGCTATGTTTATGGCGC -3'

Ala forward-3: 5'- GGCCGTGGACTGCCTGAA -3'

KA reverse-2: 5'- CCTCCTATTGCTCGGACAGC -3'

rp4 forward: 5'- AGTATCTGATGCCCAACATCG -3'; reverse 5'-

TTCCGACCAGGTTACAAGAAC -3'

PCR for rp49 was used as the internal control to evaluate the relative amounts of cDNA.

Genomic DNA extraction: The protocol was based on the method described by (Baker, 1983). 10 male flies were collected and stored on ice. 100 µl of grinding buffer was added, and the flies were ground with a pestle. Another 100 µl grinding buffer was then added, while the samples were kept on ice. Samples were then incubated at 70 °C

for 30 min, and then centrifuged for 2 min at 7500 rpm. 100 ul supernatant was then mix with 35 µl 8M KOAc and put on ice for 30 min. Samples were centrifuged for 10 min at full speed. To the supernatant 150 µl isopropanol was added and mixed gently by inverting the tubes for several times at room temperature, for 5 min. Pellets were precipitated by 10 min centrifugation at full speed. The pellets were rinsed with 70% ethanol and then centrifuged for 1 min at full speed. Supernatants were removed and water was added to pellets immediately. Dissolved pellets incubated at 65 °C with 1 µl RNAase A for 20 min. 1 µl genomic DNA was used for PCR. The amplicons were all sequenced (Cornell Life Science Core Laboratories Center) to make sure that the insertions encoded the desired peptide fragments.

Statistics: All data are presented as means ± SE. Statistical significance of differences among mean values was tested using an analysis of variance (Chi-square analysis).

2.3 Results and Discussion

Vitelline membrane protein cross-linking in eggs from CnA^{act} mutant occurs normally

Although CnA^{act} mutant females lay eggs, those eggs never hatch. To examine the underlying defects in those eggs, I examined several egg activation events. The morphologies of CnA^{act} mutant ovaries and laid eggs appear normal. To investigate vitelline membrane (VM) protein cross-linking, 0-2 hr eggs laid from either CnA^{act}/+ (mutant) or TM3/+ (sibling control) females were collected on the grape juice-agar media. The number of eggs was scored before and after 2 min incubation in 50% bleach. The egg coverings of *Drosophila* become impermeable to bleach upon activation, so bleach-treated activated eggs remain intact and do not lyse. In contrast, eggs that are not activated properly will lyse within 2 min in 50% bleach.

In this experiment, 97.5% of TM3/+ (N=499) eggs were resistant to 50% bleach. 96.2% of CnA^{act}/+ (N=788) eggs were also resistant to bleach. Thus, VM proteins appear to cross-link normally in CnA^{act}/+ mutants relative to control, since the difference between control and CnA^{act} mutants is not statistically significant (X^2=0.009, p>0.05, Figure 2.1 and Table 1). This result is consistent with observation of normal bleach-resistance in eggs of *sra* mutants, indicating that VM cross-linking may not regulated by CN. It also agrees with previously studies that VM modification can occur independently of other known egg activation events, such as *bcd* mRNA translation and other maternal transcripts' degradation (Page and Orr-Weaver, 1996; Tadros et al., 2003; Tadros and Lipshitz, 2005).

Embryos from CnA^{act} mutant females arrest early

To further investigate the defect that causes the eggs of CnA^{act} females to fail to hatch, I examined another aspect of egg activation: cell cycle progression. I used immuofluorescence microscopy to image and assay the phenotypes of these eggs. TM3/+ control and CnA^{act} mutant virgin females were mated to w^{1118} wild type males, and 0-2 hr and 2-4 hr laid eggs were collected. Eggs were fixed and stained with the mouse anti-α-tubulin antibody to reveal the spindles, and with propidium iodide to visualize the chromosomes.

In *Drosophila* embryogenesis, the two meiotic divisions of the eggs nucleus are completed 25 min of egg activation. Male and female pronuclei are formed and together enter into mitotic cell cycle. After around 9 syncytical mitotic divisions, some nuclei start to move peripherally toward the oocyte membrane; a few stay centrally to form vitellophages. About 1.5 to 2 hr after, the peripheral blastoderm nuclei become very clear and discernible. In Figure 2.2A and 2.2B, most of the control embryos progress normally through syncytical divisions and show normal orderly blastoderm nuclei localization. In contrast, very few eggs from CnA^{act}/+ females process into

Figure 2.1 Presence of constitutively-active form of calcineurin A subunit (CnA^{act}) does not affect vitelline membrane (VM) cross-linking. Embryos from CnA^{act} females have normal bleach resistance compare with control. The control here is embryos laid by TM3/+ siblings of CnA^{act} females. Numbers are listed in Table 1. ($p>0.05$, Chi-square analysis).

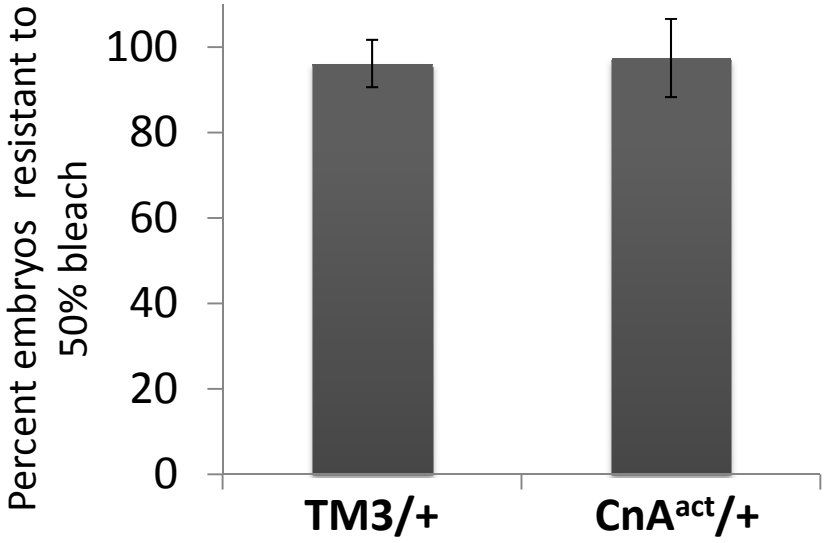

Percent embryos resistant to 50% bleach
100
80
60
40
20
0
TM3/+
CnA^act/+

Table 1 *In vivo* VM egg-covering hardening. 0-2 hr eggs were collected from either control or mutant females. The eggs were then put into 50% bleach for 2 min. Percentage indicates the number of eggs that resistant to bleach. (X^2=0.009, p>0.05)

Female genotype	No. of Eggs	Resistant to bleach	Percentage
TM3/+	512	499	97.5
CnA^{act} /+	815	788	96.2

mitotic stages. 47/53 of 0-2 hr CnA^{act} embryos were arrested during meiotic stages and did not undergo any mitotic division (Figure 2C and 2D, Table 2). The other 6/53 embryos appeared to complete meiosis but showed defects in syncytical divisions (Figure 2D and 2E, Table 2). To verify the arrest in later stages, I also examined 2-4 hr laid eggs from both control and mutant mothers (Table 2). Consistent with what I observed in 0-2 hr eggs, eggs laid by control females developed as expected for 2-4 hr embryos: most were undergoing division and gastrulation. In contrast, none of the eggs laid by CnA^{act} females progressed into gastrulation. These indicate that mutant eggs fail to undergo mitotic division, and these defects probably affect early meiotic stages.

Polyadenylation of bicoid mRNA is normal in CnA^{act} mutant embryos

Eggs from *sra* mutant females fail to fully polyadenylate *bicoid (bcd)* mRNA (Horner et al., 2006). Therefore, I wanted to determine whether direct regulation of CN also modulates the poly(A) tails of *Drosophila* maternal mRNAs such as *bcd* during egg activation. Translation of *bcd* mRNA upon egg activation is necessary for the anterior development of *Drosophila* embryos (Driever and Nusslein-Volhard, 1988). Maternal *bcd* mRNA remains untranslated in mature oocytes; upon activation, the length of *bcd* mRNA poly(A) tail is extended and BCD translation can occur (Salles et al., 1994; Surdej and Jacobs-Lorena, 1998).

To determine if CnA^{act} mutant affects polyadenylation of maternal *bcd* mRNAs, I used a PCR-based poly(A) test (PAT) (Salles et al., 1994) to detect the length of poly(A) tail before and after egg activation. Ovaries and 0-2 hr embryos were collected from CnA^{act}/+ mutant females. Eggs from sibling females with one copy of the TM3 balancer chromosome (TM3/+) were used as controls. In TM3/+ control ovaries, the *bcd* poly(A) tail is about 70 nt long; upon activation, its length increases to about 140 nt (Figure 2.3). The smeared bands in embryos are because they are a mixture of embryos from different stages, which contain different poly(A) tail lengths (Cui et al.,

2008). *Bcd* poly(A) tail lengths of these embryos from CnA^{act}/+ mutant females show no difference from controls: the *bcd* poly(A) tail length also changes from ~70 nt in ovaries to ~140 nt in activated eggs.

These results contrast with those reported for embryos from *sra* mutant females, which shows defective poly(A) tail elongation (Horner et al., 2006). This indicates that *sra* may not solely act as an inhibitor of CN in modulating poly(A) tail extension, because if *sra* inhibits CN, then the phenotypes of eggs laid by a CnA^{act} mutant should be similar to those laid by a *sra* null mutant. It is difficult to interpret my finding of polyadenlyation modulation in both *sra* and CnA^{act} eggs upon activation. Also, our present understanding of CN in *Drosophila* egg activation cannot fully explain how *sra*/CN regulates the extension of poly(A) tail. Mochida and Hunt (2007) postulate that CN activity transiently increases after Ca^{2+} injection and then decreases around the time of activation in *Xenopus*, suggesting that it is critical to regulate CN activity. The model presented by Takeo et al. (2010) indicates that phosphorylation states of *sra* affect activation and inhibition of CN. Therefore, I hypothesize that SRA functions as both inhibitor and activator of CN. In *sra* null mutant, CN cannot be properly regulated to control polyadenylation, thus causes inability of poly(A) tail elongation and maternal mRNA translation (Figure 2.4A); in contrast CN in CnA^{act} eggs is activated all the time, and at high level. Thus, *sra* may act to inhibit/balance CN activity, and bring it back to a normal level, which then allows the progression of poly(A) tail elongation upon egg activation (Figure 2.4B). This simplified model does not exclude the involvement of other mechanisms/pathways, it simply providing one possible explanation for poly(A) tail regulation. The exact mechanisms of how the CN signal, and *sra*, relate to poly(A) tail extension upon egg activation, including what other molecules are involved in this regulation, is likely to be more complicated and awaits further exploration.

Figure 2.2 Meiotic cell cycles of CnA^{act} embryos are arrested. Immunostaining of microtubules (Green, stained with anti-α-tubulin) and DNA (Red, stained with propidium iodide). (A) 0-1 hr control embryos undergo mitotic divisions, and blastoderm nuclei were arranged in an orderly fashion under the plasma membrane. (C and E) fertilized eggs from CnA^{act}/+ mutant mothers were defective in mitotic cell cycle progressions. (B, D and F) images zoom in from (A, C and E), respectively. The majority of eggs from CnA^{act}/+ mothers have no blastoderm nuclei as in (C) and (D). For (E) and (F), mutant eggs appear to complete meiosis, but somehow defect in syncytial divisions. Numbers are listed in Table 2. Scale bar: 10 μm.

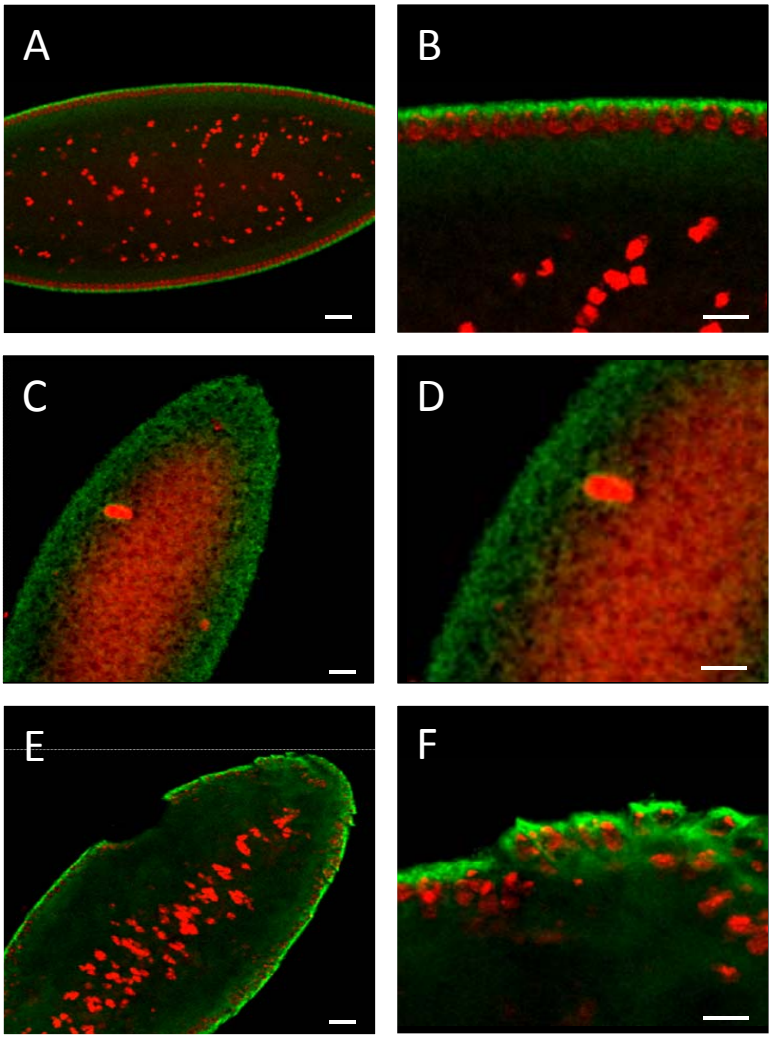

A
B
C
D
E
F

Table 2 Cell cycle arrest in developmental stages of 0-2 and 2-4 hr eggs.

Laid eggs were collected and fix from either control or mutant females. The eggs were then stained with propidium iodide and mouse anti-α-tubulin. Stages were assayed based on embryo configuration and chromosome states.

Genotype (0-2 hr)	Mitotic progression	Meiotic arrested
TM3/+	30/30	0/30
CnA^{act}/+	6/53	47/53

Genotype (2--4 hr)	Mitosis progression	Gastrulation	Meiotic arrested
TM3/+	5/7	2/7	0/7
CnA^{act}/+	1/7	0/7	11/12

Figure 2.3 *bicoid* mRNA polyadenylation appears normal in embryos from CnA^{act} mutant females. Total RNA was extracted from ovaries and 0-2 hr embryos from female flies of the indicated phenotypes. TM3/+, the sibling control. The changes of poly(A) tail lengths were examined with PCR-based PAT assays as described in the text. Tails lengths were estimated based on molecular size marker. Poly(A) tail length of *bcd* mRNA in CnA^{act} (left) appears the same as in control (right).

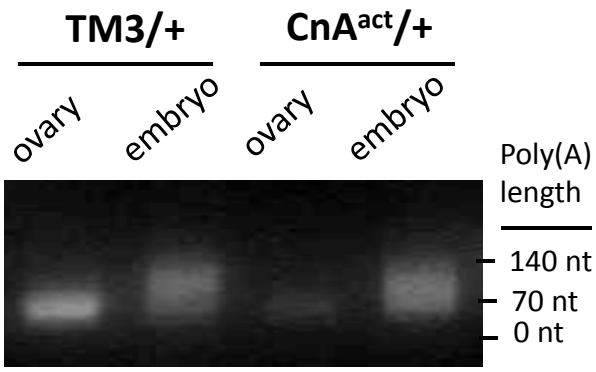
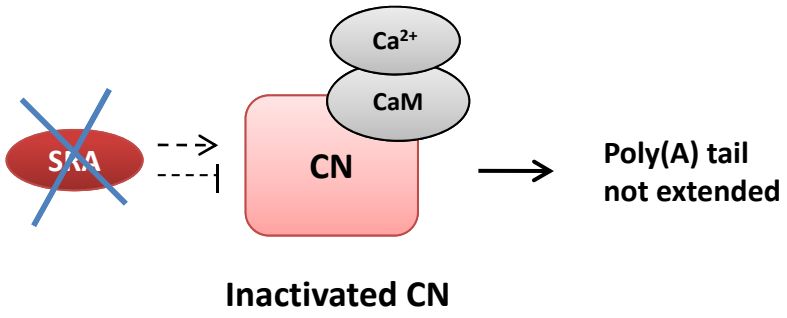

TM3/+
CnA^act/+
ovary
embryo
ovary
embryo
Poly(A)
length
140 nt
70 nt
0 nt

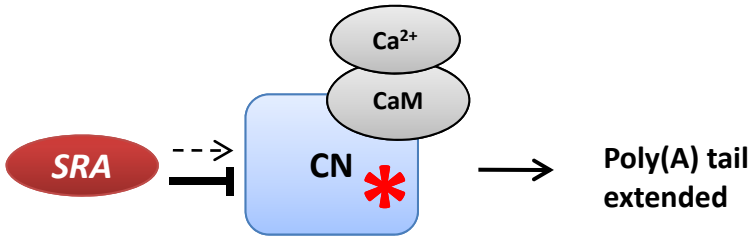
Figure 2.4 A model for poly(A) tail regulation by CN activity in *sra* and CnA^{act} mutants. (A) Null mutations in *sra* gene cause the inability to activate or repress CN. The failure of CN regulation during *Drosophila* egg activation leads to inability of poly(A) tail extension and mRNA translation. (B) In CnA^{act} mutants, CN is activated all the time. However, a normal poly(A) tail addition upon activation was observed, suggesting that CN activity may be reduced/regulated by *sra* (or by other signaling pathways) to a normally functional level before or upon egg activation. And this inhibitory regulation is essential for modulation of *Drosophila* polyadenylation.

A



Inactivated CN

B



Activated CN

MAPK is dephosphorylated prematurely in mature oocytes of CnA^{act} mutant

In *Drosophila,* levels of active- (phosphorylated-) forms of Mitogen-Activated Protein Kinase (MAPK) are highly elevated in mature oocytes. The activity/phosphorylation levels decrease upon activation without changing total amount of MAPK protein (Sackton et al., 2007). There are three MAPKs in *Drosophila:* extracellular signal-related kinase (ERK), Jun N-terminal kinase (JNK) and p38. ERK, JNK and possibly p38 all show dephosphorylation upon egg activation. The changes of MAPK activities between mature oocytes and activated embryos suggest that MAPK plays an important role in regulating *Drosophila* egg activation.

Studies in sea urchin eggs show that inactivation of MAPK activity is linked to, and likely caused by, activation of a phosphatase (Kumano et al., 2001), and there are at least two phosphatases involved in MAPK inactivation in *Xenopus* (Sohaskey and Ferrell, 1999). Though those phosphatases remain unidentified, there is some evidence suggesting association between the phosphatase calcineurin (CN) and the MAPK pathway. For example, the activation of CN leads to dephosphorylation of p38 in mouse heart (Lim et al., 2001). It is still unclear how CN interact with MAPK, but CN may act to dephosphorylate MAPK as it does for mammalian nuclear factors of activated T cells (NFATs) (Aramburu et al., 1998; Gwack et al., 2007; Jain et al., 1993). If so, the constitutively expressed CN should prematurely dephosphorylate (inactivate) MAPK regardless upstream signals. Therefore, I asked whether MAPK phosphorylation was affected in CnA^{act} mutant, and whether this affects and causes egg activation in *Drosophila* oocytes.

To determine if CnA^{act} affects MAPK activity at or before egg activation, I examined the amount of phospho-ERK in oocytes or embryos of CnA^{act}-expressing females, relative to oocytes or embryos of the TM3/+ control sibling females. In the TM3/+ control, levels of phospho-ERK are high in mature oocytes and low in

activated eggs, consistent with the decrease of MAPK activity upon activation reported by Sackton et al. (2008) (Figure 2.5A). In contrast, levels of phospho-ERK in CnA^{act} mutant mature oocytes are low, approximately at levels comparable to those in control embryos; the levels of phospho-ERK do not differ between mutant mature oocytes, mutant embryos and control embryos (Figure 2.5A and Figure 2.5B). This suggests that ERK (MAPK) is prematurely dephosphorylated in oocytes by constitutively-active CN in CnA^{act} females, and thus that activation of CN normally play a role in down-regulating ERK (MAPK) activity during oogenesis. Unfortunately, I was unable to obtain clear results for the phospho-levels of JNK or p38 (CnA^{act} and control) (data not shown).

This result raises the question about the relationship between CN and MAPK regulation. Takeo et al. (2010) shows that CN is positively regulated by *sra* in *Drosophila* (Takeo et al., 2010). In one simple hypothesis, if *sra* activates CN, and CN causes MAPK dephosphorylation, then I should not get MAPK dephosphorylation in *sra* mutants; in contrast, I should get premature inactivation of MAPK in CnA^{act} mutants. My result of CnA^{act} mutants agrees with this hypothesis and suggests that CN is responsible for MAPK dephosphorylation. Intriguingly, Sackton et al. (2007) presented data against this hypothesis: These authors found that *sra* does not affect MAPK levels during *Drosophila* egg activation. Therefore, the regulation of MAPK could be more complicated. For example, If *sra* sometimes activates CN, and sometimes represses it (or other pathways are redundant for *sra*/CN regulation), then the inactivation of *sra* may not have any effect on MAPK regulation. As noted earlier, CamKII is associated with at least one egg activation event in *Xenopus* (Lorca et al., 1993). It is possible that *Drosophila* CamKII may coordinate with CN and also plays a role in MAPK dephosphorylation (Figure. 2.6). In this model, embryos from *sra* mutants would have normal MAPK phosphorylation/ dephosphorylation patterns

because CamKII compensates for CN function during egg activation; while in CnA^{act} mutants, constitutively-active CN leads to dephosphorylation of MAPK even before egg activation.

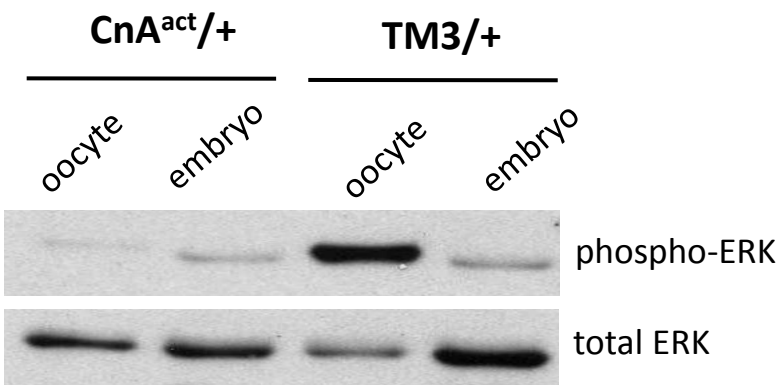
Inhibitors of CaM and CamKII did not affect female fertility

In egg activation studies, two proteins that are regulated by Ca^{2+}-CaM are of particular interest: the protein phosphatase calcineurin (CN) and the Ca^{2+}/calmodulin-activated kinase II (CamKII) (Griffith, 2004). As discussed above, CN is important for several egg activation events in *Drosophila* (Horner et al., 2006; Takeo et al., 2006), and in *Xenopus* (Mochida and Hunt, 2007). CamKII has been shown to be essential for several egg activation events in mouse and *Xenopus* as well (Ducibella et al., 2006). Vanessa Horner (unpublished data) obtained preliminary data suggesting that CamKII activity might increase upon egg activation in *Drosophila***.** She assessed the amount of phospho-T287, which is a conserved residue on CamKII that is auto-phosphorylate itself when CamKII is activated. Using antibody specific to that site, she found that pT287 levels increased upon activation. Calmodulin (CaM) is pivotal in regulating both CN and CamKII. Therefore, study of the roles of CaM and CamKII is necessary for understanding how Ca^{2+} functions in *Drosophila* egg activation.

Several genetic approaches can normally be used to explore the function of genes in egg activation. For CaM and CamKII, however, there are many difficulties with these approaches. First, null mutations in both CaM and CamKII genes are lethal. Both proteins are involved in many different molecular processes throughout the life of the organism (Johnson et al., 2002; Wang et al., 2003). To obtain a phenotype, one would need clones in the germline. However, there are problems for germline clonal analysis. *Drosophila* has only one CamKII gene but it is located on chromosome 4 (Andruss et al., 2004). Chromosome 4 is very tiny and no FLP site has been

Figure 2.5 Change in levels of phospho-ERK levels during *Drosophila* egg activation. The levels of active (phospho‑) forms of ERK and ERK protein levels (total ERK) were examined with western blotting. Total ERK protein is an internal loading control. TM3/+ are the siblings of the CnA^{act} mutant females used here, and serve as controls. In control, levels of active ERK decrease when the oocytes are activated and transition into embryos (compare 3rd lane and 4th lane of Fi.g A). Phospho-ERK levels are severely decreased in mutant oocytes compared with control oocytes (compare 1st and 3rd lanes of Fig A), and there is no significant change of active ERK levels between mutant oocytes and embryos (compare 1st and 2nd lanes of Fig. A). B, quantification of phosphor−ERK levels relative to total ERK activity. Data are presented as means ± SE. Western blots were scanned and were analyzed by Image J software. Levels of phospho‑ERK were normalized to total ERK. Results shown are the average of four biological repeat experiments. Levels of phospho−ERK show around 6-fold decrease during the transition from control oocytes to control embryos. However, the levels of phospho-ERK stay the same, and low, in both CnA^{act} mutant oocytes and embryos. Moreover, in mutant oocytes or embryos, levels of phospho-ERK are as low as the control embryos, indicating that CnA^{act} mutant affects the states of phosphorylation of MAPK pathway.

A


CnA^act/+
TM3/+
oocyte
embryo
oocyte
embryo
phospho-ERK
total ERK

B

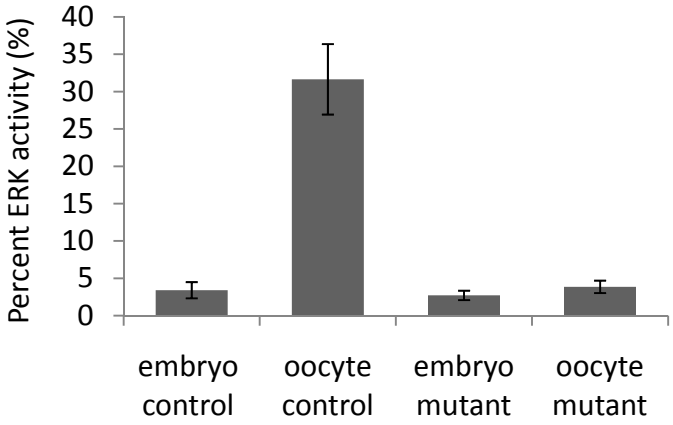
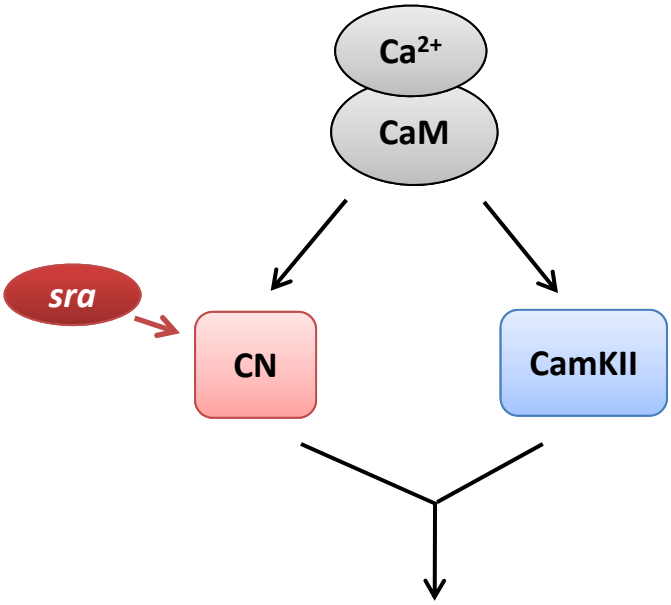

Percent ERK activity (%)
40
35
30
25
20
15
10
5
0
embryo control
oocyte control
embryo mutant
oocyte mutant

Figure 2.6 A diagram for proposed MAPK dephosphorlation regulation by Ca^{2+} signaling pathways in *Drosophila*. SRA activates CN during female meiosis (Takeo et al. 2010). Upon egg activation, Ca^{2+}/CaM , together with *sra,* bind to and activates CN, which leads to the dephosphorylation of MAPK (ERK). In *sra* mutants, CN cannot be positively regulated by *sra,* which may keep CN silent at egg activation. However, in this model a Ca^{2+}/CaM triggered CamKII pathway compensates for CN, in the regulation of MAPK, leading to normal MAPK activity. In CnA^{act} mutants, CN activity is high before or after egg activation. The overall signaling causes a premature dephosphorylation of MAPK in CnA^{act} mature oocytes.


Ca2+
CaM
sra
CN
CamKII
MAPK dephosphorylation

engineered within it. Thus germline clonal analysis has not been possible for CamKII. One gene in *Drosophila* encodes CaM (Doyle et al., 1990), and germline clones of a null mutant were created by Andruss et al. (2004) using FLP-dominant female sterile technique (Perrimon et al., 1996). Surprisingly, they found that CaM-deficient germline clones do not totally eliminate CaM from early embryos (Andruss et al., 2004). The authors hypothesized that this could due to movement of CaM into germline cells from other cells. Thus, no CaM mutant germline clones could be obtained. I sought to use a different approach to test the role of CaM and CamKII in the germline - expressing peptides that inhibited of those proteins.

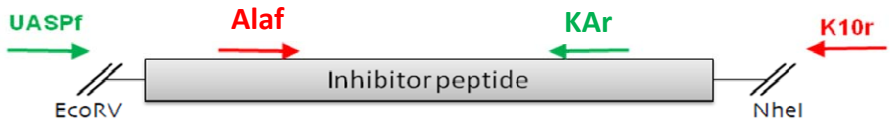
CaM is the major intracellular receptor of Ca^{2+}. It contains four EF-hand motifs, which bind to Ca^{2+} ion. After Ca^{2+} binding, a conformational change causes hydrophobic methyl groups on CaM's methionine residues to become exposed. These hydrophobic surfaces bind to Basic Amphiphilic Helices (BAA helices) on the target protein and remove autoinhibitory domains from the target proteins' catalytic site. The interactions are critical for activation of CaM's target proteins (Weinstein and Mehler, 1994). Studies showed that synthetic peptides analogs of this regulatory domain of CaM are effective inhibitors of CaM activity (McCarron et al., 1992; VanBerkum and Means, 1991). VanBerkum and Goodman (1995) generated a transgene that expressed one of such peptides, kinesin-antagonist (KA), in *Drosophila* that binds to and blocks CaM. They showed that KA disrupted CaM binding to target proteins in *Drosophila* neurons (VanBerkum and Goodman, 1995). So, to inhibit CaM in oocytes, we sought to express KA in the germline.

CamKII is a major substrate of CaM which is composed of an N-terminal catalytic domain, a regulatory domain, and an association domain. The catalytic domain is autoinhibited by its regulatory domain until the activation by Ca^{2+}/calmodulin. Once activated by Ca^{2+}/calmodulin, CamKII autophosphorylates

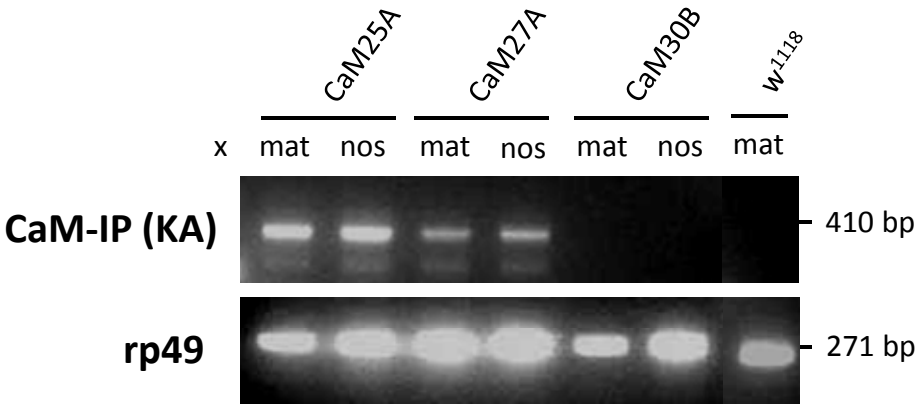
itself (Lisman, 1985). Peptide analog of this autoinhibitory domain were created in *Drosophila* based on mouse CamKII α-subunit: alanine (Ala) inhibitory peptide (Griffith et al., 1993). This peptide was shown to inhibit CamKII action in neurons, by Griffith lab (Griffith et al., 1993). So, to inhibit CamKII, we sought to express Ala in oocytes.

Before I joined the Wolfner lab, they had acquired flies carrying UAS-KA and UAS-Ala from M. VanBerkum and L. Griffith, respectively. An undergraduate, Kritika Kumar and three graduate students (K. Sackton, J. Cui and V. Horner) had used PCR to move the sequences from pUAST to germline expression vector pUASP, and they had generated transgenic flies carrying the UASP-peptide expression constructs. They produced three independent UASP-KA and nine UASP-Ala lines. I mated females from those lines with males carrying either *nos*-Gal4 or *maternal tubulin (mat)*-Gal4 germline-specific drivers; I obtained similar levels of germline expressions with both drivers (Figure 2.7A). I used RT-PCR to examine expression of the peptide-encoding RNA in all 12 lines. I found that all CamKII lines exhibit and express Ala inhibitory peptide (expression data shown in Figure 2.7A and B). However, only two of the three CaM lines (CaM 27A and CaM 25A) appeared to express the KA fragment (data not shown). Unfortunately, both lines expressed an RNA encoding a truncated KA peptide, having only 20 a.a. compared with full length 41 a.a. (KA peptide: QKDTKNMEAKKLSKDRMKKYMARRKWQKTGHAVRAIGRLSS; truncated KA shown by the underline). As expected by those not expressing full KA sequence and thus likely not inhibiting CaM, CaM27A and CaM25A did not affect female hatchability (Figure 2.7A and Figure 2.8). All nine CamKII inhibitor lines that express fully Ala inhibitory peptides, showed no effect on *Drosophila* female hatchability as well (Figure 2.7B and Figure 2.8). This negative result could due to many reasons: First, the CamKII pathway may not be involved in Ca^{2+} triggered egg activation events.

Figure 2.7 Presence of CaM and CamKII inhibitory peptides. (A) Inhibitor peptide (IP) females were mated with either *nos*-Gal4 (nos) or *maternal tubulin*-Gal4 (mat) males. Total RNA was extracted from the ovaries of 3-5 day-old female progeny. mRNA were reversed transcribed into cDNA for PCR analysis. Rp49 is a positive control for cDNA quality. w^{1118} x *mat* was used as a negative control for IP. Three CaM-inhibitory (UASP-KA) lines (25A, 27A and 30B) driven by two different drivers were tested, as indicated on the figure. Lines 25A and 27A show KA expression, but these expressions are incomplete in their sequences. The primer set used for examining KA peptide was UASPf and KAr (Green). Sequences of primers were listed in the text. No difference detected between two germline specific drivers. (B) The same experiment with CamKII IP lines. The figure shows only two out of nine CamKII-inhibiotry (UASP-Ala) lines driven by mat driver. The primer set used for examining Ala peptide was Alaf and K10r (Red). Sequences of primers were listed in the text.


UASPf
Alaf
KAr
K10r
Inhibitor peptide
EcoRV
NheI

A


CaM25A
CaM27A
CaM30B
w1118
x
mat
nos
mat
nos
mat
nos
mat
CaM-IP (KA)
410 bp
rp49
271 bp

B

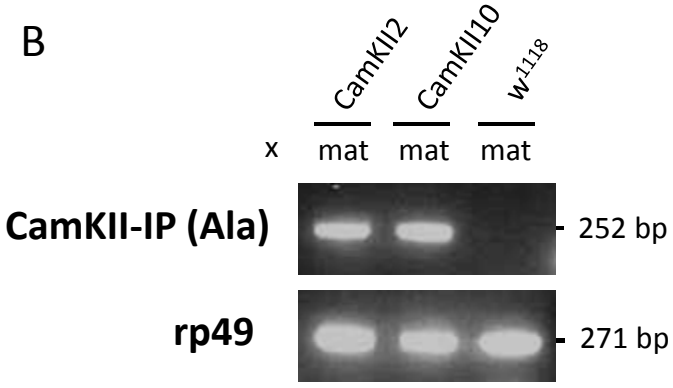
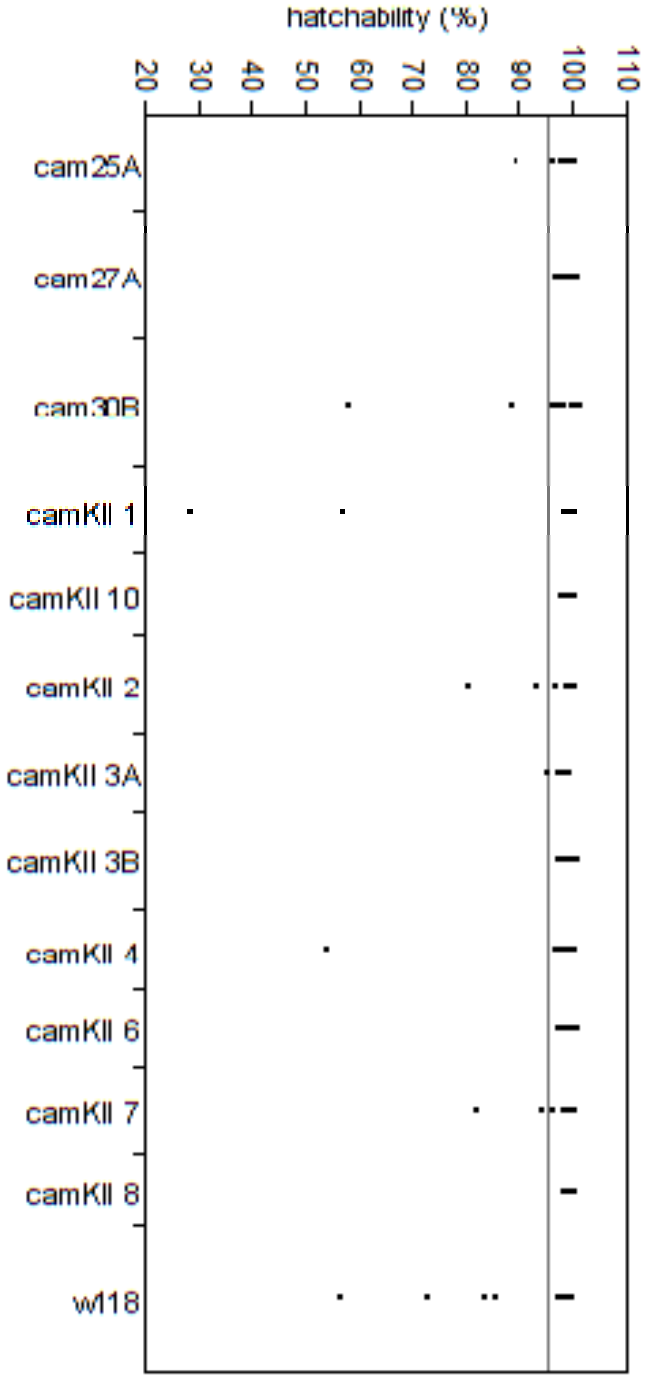

CamKII2
CamKII10
w1118
x
mat
mat
mat
CamKII-IP (Ala)
252 bp
rp49
271 bp

Figure 2.8 Assay of *Drosophila* hatchability in CaM and CamKII inhibitory peptide lines. Inhibitory peptide (IP) virgin females (*nos*-GAL4; UASP-KA or Ala) or control w^{1118} virgin females were mated with w^{1118} males. Single mated females were transferred into individual new vial every 24 hr for 5 days. Eggs laid by those females were scored after each transfer, and progenies were also counted. Hatchability rate was calculated by the formula: number of progeny/numbers of eggs. w^{1118} is a positive control. There is no significant difference in hatchability of eggs between IP lines and control. The experiment was repeated with the *maternal tubulin*-GAL4 driver, and results from that experiment were like those shown here. N=6.


hatchability (%)
20
30
40
50
60
70
80
90
100
110
cam25A
cam27A
cam30B
camKII 1
camKII 10
camKII 2
camKII 3A
camKII 3B
camKII 4
camKII 6
camKII 7
camKII 8
w1118

Alternatively, other regulators or molecular pathways may also act redundantly with CamKII and may thus compensate for any inhibitions of CaM and CamKII. Third, Ala peptides may not have been expressed at a sufficient level to totally block CamKII function. This is likely because a low level inhibition of Ala peptide (~15-25%) was detected previously (Griffith et al., 1993). CamKII pathway is critical for many molecular processes, so the inhibitory level may not be enough to disrupt CamKII's function. Thus, remaining CamKII could still respond to Ca^{2+} signaling and to regulate downstream mechanisms.

2.4 Conclusion

I have shown that eggs laid by CnA^{act}-expressing mothers are defective in several but not all aspects of *Drosophila* egg activation events, suggesting that some Ca^{2+} signals for activation are probably partially transduced through calmodulin(CaM)-calcineurin(CN). I showed that CN is necessary for cell cycle completion and likely for dephosphorylation of MAPK upon egg activation. However, poly(A) tail extension of *bcd* mRNA is not affected in CnA^{act} mutants, even though mutations of a CN regulator, *sra*, affected their polyadenlyation. CnA^{act} mutations cause an early cell cycle arrest in *Drosophila*. Although the stage of the arrest is still unclear, immunofluorescence data suggest that the majority of egg laid by CnA^{act} mutant do not process beyond the syncytical stage and do not undergo mitotic division. Levels of active- (phospho-) ERK are dramatically low in CnA^{act} mutant oocytes, suggesting the possible role of activated CN to prematurely inactivate ERK in oocytes, and the normally high levels of MAPK activity seen in oocytes require active repression of CN at this stage.

Though a recent study showed that CamKII inhibits CN in rat heart muscle *in vitro* (MacDonnell et al., 2009), the relationship between these two pathways in

modulating *Drosophila* egg activation needs to be studied further. I tried to inhibit CaM and CamKII functions in the female germline using inhibitor peptide-expressing lines generated previously by our lab. I showed that the CaM lines expressed mRNA for a truncated peptide that might not be a good inhibitor, and the correct lines would need to be created by using this method to study CaM. For CamKII, I showed that although the expressed transgene was of correct sequence, there was no hatchability effect of its expression. Thus, further experiments will be needed to determine whether CamKII (or CaM) play roles in egg activation.

CHAPTER 3

IDENTIFICATION AND CHARACTERIZATION OF *PRG* GENE IN DROSOPHILA EGG ACTIVATION

3.1 Introduction

During oogenesis, oocytes undergo meiosis and develop into mature oocytes. Egg activation triggers the initiation of embryogenesis, including cell cycle progressing and DNA synthesis. Early embryonic development requires the products of maternally-loaded RNAs, but translation of most maternal RNAs is masked during late oogenesis. Other maternal transcripts are destabilized and degraded during early embryogenesis, allowing the zygotic products to take control of embryo development (Tadros et al., 2003).

In *Drosophila*, maternal transcripts that are destabilized during embryogenesis (Bashirullah et al., 1999; Edgar and Datar, 1996), include *Hsp70, twine* and *nanos*. Maternally-loaded Smaug (SMG) protein, an RNA-binding protein, mediates most of the destruction of maternal RNAs in early *Drosophila* embryos (Tadros et al., 2007). After being activated by Pan Gu kinase, SMG binds to its target transcripts, which contain a *cis*-element SMG-recognition site. SMG recruits CCR4/POP2/NOT-deadenylase complex to these transcripts, causing the transcripts' deadenylation. However, the details of the process are unclear at the molecular level (Tadros and Lipshitz, 2009).

To dissect the process of maternal mRNA destabilization, Tadros and Lipshitz (2003) mounted a genetic scressn for X-linked mutations that prevented destabilization of maternal mRNAs. The screen identified genes involved in RNA metabolism. One of these genes, *wisp*, was subsequently shown to encode a poly(A) polymerase that is

essential in late oogenesis and for egg activation. This Chapter concerns the identity of another gene identified in that genetic screen: ***prage* (*prg*)**.

Embryos from *prg* mutant mothers fail to destabilize maternal transcripts. These embryos get fertilized but never hatch. Since these phenotypes suggest that *prg* must be defective in egg activation, I started to characterize egg-activation phenomena in embryos produced by mutant females in either of two *prg* alleles (prg^{16A} and prg^{32}). I found that eggs from *prg* mutant mothers do not cross-linked properly.

Because my data, and data from Tadros et al. (2003) and from Jun Cui (Thesis 2010) indicated that the *prg* protein likely played a role in egg activation, it was important to know the identity of the *prg* gene's product. The *prg* gene had been mapped to a relatively large range (1B4-1E2) of the X chromosome by Tadros et al. (2003). Jun Cui then narrowed down the position of *prg* by complementation analysis with *prg* mutants and deletions within the 1B4-1E2 region. Finally, he narrowed down the *prg*-containing region to one that contained only 10 genes. He sequenced each of these genes in the prg^{16A} and prg^{32} mutant strains. One gene, CG14801, which encodes a predicted exonuclease, contained lesions in both mutant strains, and both mutations encoded truncated versions of CG14801. This suggested that *prg* corresponded to this gene. According to the latest annotation of the *Drosophila* genome from *Flybase* (http://www.flybase.org), CG14801 transcript contains six predicted isoforms with alternative splicing at the 5' end. Both *prg* alleles have single base pair changes in the common exons near the 3' end of the transcript which are shared by all six isoforms. I report here complementation tests with a P-element insertion into CG14801 that confirm that this gene is *prg*. I then determined *prg* expression by RT-PCR, showing that it is expressed in adults of both sexes and developing embryogenesis. Finally, I report my efforts to generate an anti-PRG antibody, which I plan to use to examine PRG protein expression and, if possible, its localization.

During oocyte maturation, polyadenylated mRNAs are stabilized by forming a closed loop, which binds 3' poly(A) tail to the 5' cap-binding complex (Tadros and Lipshitz, 2005). The interaction can be disrupted by deadenlyation of transcripts during oocyte maturation, resulting in uncapping of the mRNAs. Such mRNAs will be targeted for degradation by a 5' to 3' exonuclease. Alternatively, mRNAs can also be degraded by a 3' to 5' exonuclease after deadenylation. In *Xenopus,* deadenlyation was found to be regulated by xPARN, a deadenylase that homologue of human PARN (Copeland and Wormington, 2001; Dehlin et al., 2000). *Drosophila*, however, doesn't have a PARN homolog, and it is suggested that another deadenylase (CCR4) plays an analogous role (Semotok et al., 2005; Tadros et al., 2007). It is not very clear whether other mechanisms/molecules are involved in maternal mRNA destabilization in *Drosophila* egg activation. The finding and characterization of *prg* gene as a possible RNA exonuclease may shed light on the mechanism of maternal mRNA degradation in *Drosophila*.

Materials and Methods

Fly stocks: Oregon-R P2 and w^{1118} were used as wild type stocks. prg^{16A}/FM6 and prg^{32}/FM6 (Tadros et al., 2003) were kind gifts from W. Tadros and H. Lipshitz (Hospital for Sick Children, University of Toronto, Canada). Deficiency strains *Df(1)BSC719/Binsinscy* and three P-element insertion lines, P{Mae-UAS.6.11}CG42666^{GG01337}, P{EPgy2}CG42666^{EY21466} and P{XP}CG42666^{d10828} (Bloomington Stock number CG14630, CG19337 and CG22483, respectively) were ordered from the Bloomington Stock Center (Indiana University). *Df(1)BSC719/*Binsinscy virgin females were crossed with FM7c males to get *Df(1)BSC719/*FM7c flies for maintaining the stock.

Complementation test: Virgin females from each desired P-element insertion

strain were crossed with *prg* males. Fertility of female progeny carrying P{Mae-UAS.6.11}CG42666^{GG01337}/*prg*, P{EPgy2}CG42666^{EY21466}/*prg* and P{XP}CG42666^{d10828}/*prg* were scored for complementation test.

RNA extraction and RT-PCR: Total RNA was extracted from 3-5 day old adult males, adult females and 0-2, 2-4, 4-6 hr embryos collected. cDNA was synthesized as described in Chapter 2. RT-PCR was performed to determine the expression patterns of *prg* transcripts. The primer sets for the PCR were:

prg forward 5'- ATGGAGCAAATAACGAACTACTTCG -3'; reverse 5'- TCAGTCCGTCGTGGTAGTTG -3'

19337 forward 5'- GGGCGGGTAGTGGAGATA -3'; reverse upper 5'- TCGGCTGTAAACGATGCT -3'; reverse lower 5'- AGCGAATGCTCTGCGTGT -3'

rp49 forward 5'- AGTATCTGATGCCCAACATCG -3'; reverse 5'- TTCCGACCAGGTTACAAGAAC -3'

PCR for rp49 was used as the internal control to evaluate the relative amounts of cDNAs.

prage antibody preparation: A polyclonal anti-PRG antibody was generated following the procedure in (Cui et al., 2008). A full length cDNA clone of CG14801 was obtained from *Drosophila* Gold Collection (Stapleton et al., 2002). I generated an entry clone with this full length cDNA and verified its sequence. I then moved the *prg* cDNA, in frame into the protein expression vectors pDEST15 and pDEST17. This was all done by using the Gateway entry cloning system (Invitrogen). The expression clones generated fusion proteins contain N-terminal Glutathione S-transferase (GST) or 6xHis, respectively, and *prg* sequences C-terminal. The fusion proteins were verified as having gel mobilities close to expectation, with predicted molecular weights of 114.7 kDa in pDEST15 vector and 89.6 kDa for PRG in the pDEST17 vector. The GST fusion protein was either gel purified (Monsma and Wolfner, 1988) or

column purified using glutathione beads (Guan and Dixon, 1991), and then used to immunize rabbits (Cocalico Biologicals Inc., Reamstown, PA). Once these antisera are received, PRG-specific antibodies will be affinity purified from antisera as described in Cui et al. (2008) by using 6XHis PRG. If that is not possible, then following the procedure of Bertram et al. (1996), serum will first be run through a Sepharose 4B column (sigma) coupled with GST protein, to remove anti-GST and anti-PRG will then be purified over a column with GST-PRG fusion protein. The purified anti-PRG antibody will be titrated in a dilution series, and checked for specificity by testing for gel mobility differences of PRG^{16A} and PRG^{32} related to wild type.

3.2 Results and Discussion

Vitelline membranes do not cross-link properly in prg *mutant eggs*

prg mutant females are sterile. I began to characterize the activation phenotypes with standard assays presented in Chapter 2. The morphologies of dissected mutant ovaries and laid eggs are normal compare with wild type. To test whether the vitelline membrane (VM) cross-links normally in eggs from *prg* mutant females, eggs laid from mated mothers were collected for 2 hr time intervals, and these *in vivo* activated eggs were used for bleach assay. After a 2 min incubation in 50% bleach, cross-linked eggs become resistant to bleach, while non-cross-linked eggs lyse within 2 min incubation. Around 50% eggs laid by homozygous *prg* females fail to undergo VM cross-linking compare to those of heterozygous controls (detail as shown in Table 3).

In addition, I generated fusion proteins expressing full length PRG, which I purified and sent to immunize rabbits for making polyclonal anti-PRG. This generation will help us further examine *prg* during *Drosophila* egg activation. For example, the presence of PRG proteins in different embryonic stages can be examined, and the

Table 3 Eggs laid from *prg* mutant females undergo VM hardening at significantly lower levels than control. 0-2 hr eggs were collected from either ORP2 control or *prg* mutant females. The eggs were incubated in 50% bleach for 2 min. Numbers in parentheses indicate percentages, and where appropriate ± SE. (** $p \leq 0.001$)

Female genotype	Resistant/Total # of eggs (1)	Resistant/Total # of eggs (2)	Resistant/Total # of eggs (3)	Sum Resistant/Total
prg^{16A}/FM6	26/26 (100)	50/53 (94.3)	40/42 (95.2)	106/111
	**	**	**	(95.5 ± 3)
prg^{16A}/prg^{16A}	7/12 (58.3)	10/23 (43.5)	21/45 (46.7)	38/80
				(47.5 ± 7.8)
prg^{32}/FM6	65/67 (97)	32/32 (100)	56/59 (94.9)	153/158
	**	**	**	(96.8 ± 2.6)
prg^{32}/prg^{32}	19/33 (57.6)	22/38 (57.9)	18/34 (52.9)	59/105
				(56.2 ± 2.8)

(1) $X^2 = 12.5, p \leq 0.001$; prg^{32}: $X^2 = 25.6, p \leq 0.001$

(2) $X^2 = 27.7, p \leq 0.001$; prg^{32}: $X^2 = 17.5, p \leq 0.001$

(3) $X^2 = 24.5, p \leq 0.001$; prg^{32}: $X^2 = 23.4, p \leq 0.001$

effect of mutations in *wisp* and other egg activation mutants on PRG translation can be determined. If the antibody permits, we can also determine PRG's subcellular localization.

CG14801 corresponds to the* prg *gene

As described in introduction, Jun Cui showed that CG14801, the predicted *prg* gene, encodes a protein that contains a conserved exonuclease domain (*Flybase*) (Figure 3.1). In addition, mutants in *prg* alleles, prg^{16A} and prg^{32}, give rise to truncated proteins that lack the exonuclease domain. This is due to single base pair changes of *prg* alleles in the common exon regions near the 3' end of the transcript which are shared by all six isoforms (CG14801-PA shown in Figure 3.1 corresponds to protein product of CG42666-PA CDS in Supplementary Figure A.1). These results suggest that PRG might be a exonuclease whose activity might mediate maternal transcripts degradation during egg activation, and that the failure of this degradation in *prg* mutant embryos (Tadros et al., 2003) is likely due to nonfunctional PRG exonuclease.

To confirm that *prg* gene corresponds to CG14801, Rebecca Zuckerman and I carried out complementation tests of *prg* mutations, with P-element insertions in CG14801. We tested for complementation between both *prg* mutant alleles (prg^{16A} and prg^{32}) and three P-element insertion lines which are available from Bloomington Stock Center. Two insertions, P{Mae-UAS.6.11}$CG42666^{GG01337}$ (Mae) and P{EPgy2}$CG42666^{EY21466}$ (EPgy2), failed to complement for both *prg* mutant alleles. However, P{XP}$CG42666^{d10828}$ (XP) unexpectedly complemented both *prg* alleles. I confirmed, by RT-PCR with primers specific to XP line, that this line had an insertion in CG14801 (Figure 3.2). Based on *Flybase,* CG14801 contains six predicted isoforms (PE, PB, PD, PF, PC, PA). The insertions of Mae and EPyg2 are disrupted in all six isoforms, suggesting the universal effects of these two lines; however, XP is only inserted into one gene isoform (PE) (Supplementary Figure A.1). The simplest

Figure 3.1 Schematic representation of *prg* alleles (by Jun Cui). Wild-type CG14801 gene (isoform A) encodes a protein of 761 a.a. prg^{16A} and prg^{32} have nonsense mutations in the coding region that results in truncated protein of 72 a.a. and 373 a.a. respectively.

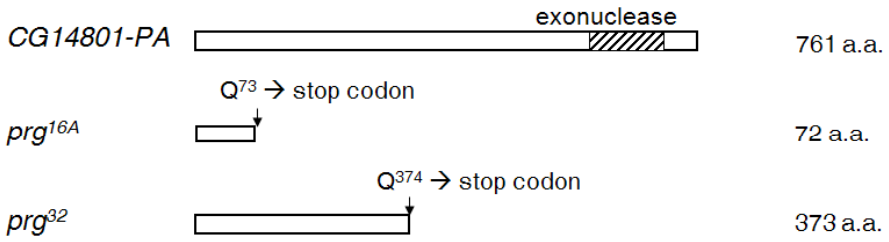
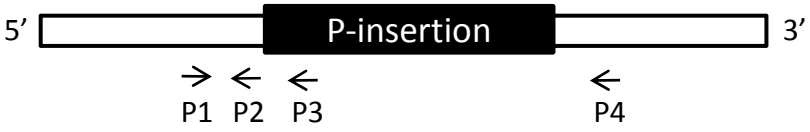
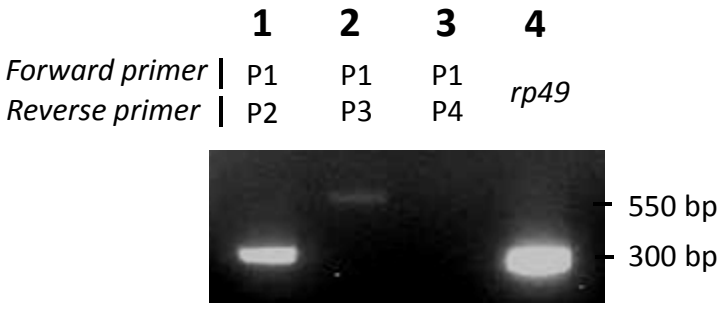
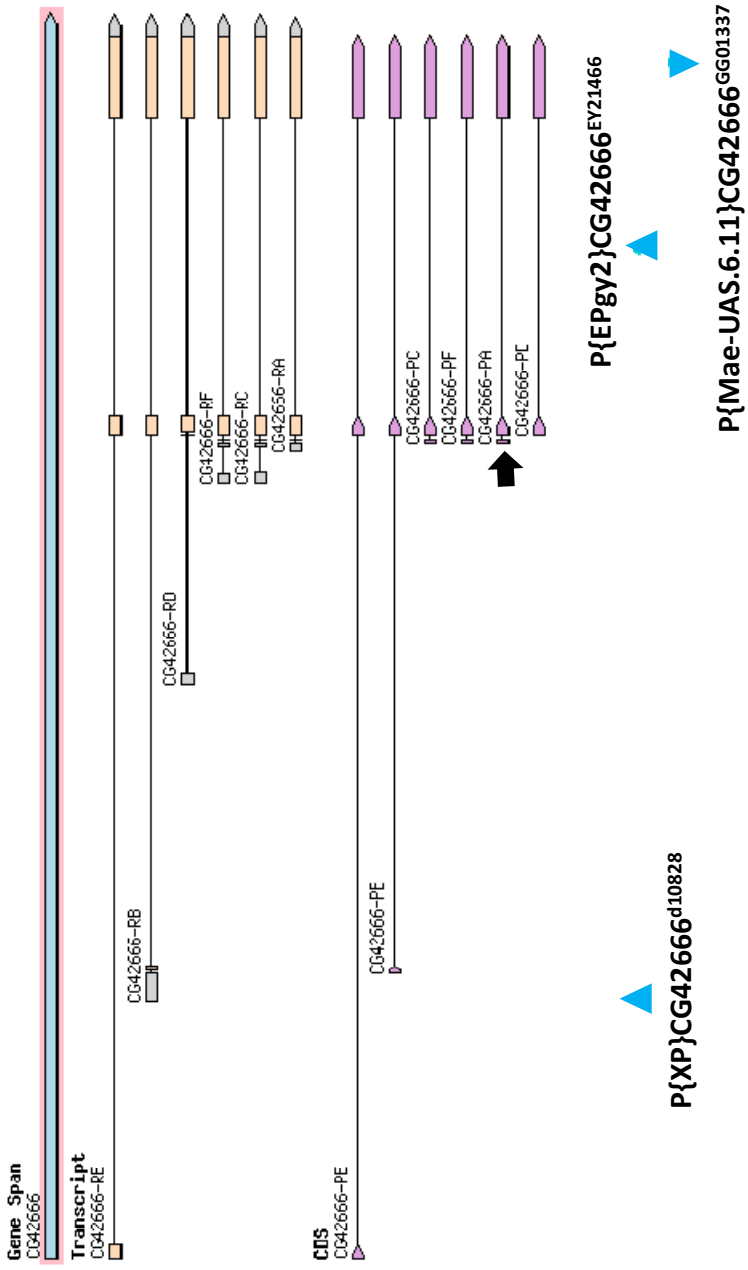

exonuclease
CG14801-PA
761 a.a.
Q73 → stop codon
prg16A
72 a.a.
Q374 → stop codon
prg32
373 a.a.

Figure 3.2 Presence of insertion, P{XP}CG42666^{d10828}, in CG14801. Gnomic DNA was extracted from ten 3-4 day old adult males and used for PCR analysis. rp49 as an internal control for evaluating the relative amounts of cDNAs. Three different combinations of *prg* primers shown in the simplified diagram (upper) were used to detect *prg* transcripts: P1&P2 - test for the presence of CG14801 gene; P1&P3 - test for the presence of P-element insertion; P1&P3 - negative control. P{XP}CG42666^{d10828} was shown to located in the predicted site, suggesting the existence of the insertion.


5’
P-insertion
3’
P1
P2
P3
P4


1
2
3
4
Forward primer
Reverse primer
P1
P2
P1
P3
P1
P4
rp49
550 bp
300 bp

Supplementary Figure A.1 A map of P-element insertions relative to *prg* gene (CG14801) structure. CG42666 is the symbol synonym of CG14801. Six isoforms (PE, PB, PD, PF, PC and PA) are shown in different lengths for both transcripts and cDNAs (CDS). The insertion sites of three P-element strains (P{XP}CG42666^{d10828}, *P*{Mae-UAS.6.11}CG42666^{GG01337} and P{EPgy2}CG42666^{EY21466}) are shown in a left to right order on the map (blue arrow head). P{XP}CG42666^{d10828} only inserted into one gene isoform (PE) as described in the text. Map modified from *Flybase* (http://flybase.org/). Protein product of CG42666-PA corresponds to CG14801-PA shown in Figure 3.1 (black arrow), suggesting that *prg* alleles are truncated in all six isoforms.


Gene Span
CG42666
Transcript
CG42666-RE
CG42666-RB
CG42666-RD
CG42666-RF
CG42666-RC
CG42656-RA
CDS
CG42666-PE
CG42666-PE
CG42666-PC
CG42666-PF
CG42666-PA
CG42666-PI
P{EPgy2}CG42666EY21466
P{Mae-UAS.6.11}CG42666GG01337
P{XP}CG42666d10828

explanation is that XP insertion does not eliminate function of CG14801 gene, if some isoforms are still expressed normally and produce functional PRG protein. Whether functions of CG14801 isoforms can compensate for each other needed further study, but the results from Mae and EPgy2 insertion lines confirm that CG14801 is the *prg* gene.

***Gene expression pattern of* prg**

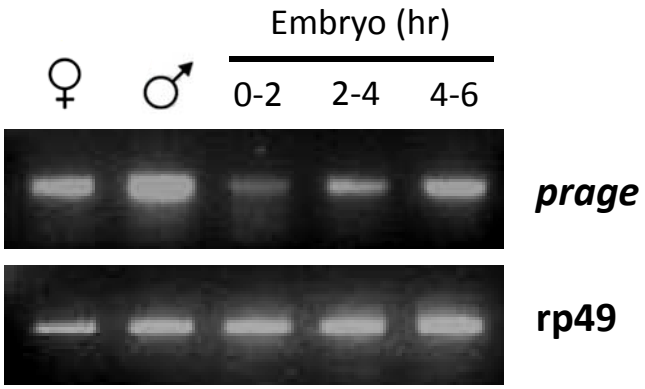
Adult flies and embryos in different stages were collected and immediately subjected to total RNA preparation as described above. Presence of *prg* mRNA was determined by RT-PCR by method described in Chapter 2. The results show a ubiquitous expression of *prg* mRNA in both male and female adult flies, and a gradual increase in level during embryogenesis (Figure 3.3). Here I focus on *prg* gene in *Drosophila* female germline, so the role of male transcripts won't be explored further.

3.4 Conclusion

In summary, Jun and I have shown that CG14801 is *prg*, and thus encodes a possible RNA exonuclease. Previous studies suggest the essential role of exonucleases in regulating mRNA degradation in eukaryotes (Kushner, 2004; Meyer et al., 2004). Specific sequences in the 3' UTR can target cytoplasmic mRNA for deadenylation, followed by either exosome (3' to 5') degradation or exonuclease (5' to 3') decapping/degradation (Houseley and Tollervey, 2009).

RNA exonuclease 1 (REXO1) is a 3' to 5' exonuclease which belongs to the REXO1/REXO3 family. REXO1 is required for RNA editing and maturation in yeast (Frank et al., 1999; Nariai et al., 2005; van Hoof et al., 2000). However, REXO1 homolog in human and mouse have no detectable function (Tamura et al., 2003). As noted above, the RNA degradation mechanisms are somehow different in different organisms. For example, CCR4 deadenylase was found to act in both *Drosophila* and yeast, but not in mouse or human. Therefore, it is likely that RNA exonuclease *prg*

Figure 3.3 Expression patterns of *prg* transcripts. Total RNAs were extracted from 3-5 day old wild-type male and female adult flies or laid embryos with desired time. mRNAs were reverse transcribed into cDNA for PCR assay as described above. rp49 as an internal positive control. *prg* transcripts were expressed in all samples examined. The amount of transcripts increased throughout the embryogeneiss.


Embryo (hr)
♀
♂
0-2
2-4
4-6
prage
rp49

may also play a role in RNA editing or RNA decay in *Drosophila* as that in yeast. Though very little is known about the mechanism of mRNAs destabilization/degradation in *Drosophila* egg activation, the deadenylation was shown to be responsible for the elimination of over 1600 maternal transcripts, and is essential for maternal-to-zygotic transition (Tadros et al., 2007). Therefore, the study of *prg* may help us to understand how this RNA exonuclease acts to affect *Drosophila* egg activation.

I found that *prg* is expressed in both adult males and females, and that its expression gradually increased with embryonic development. In conjunction with data from Tadros et al., my results show that *prg* defective in at least two egg activation events, maternal transcript degradation and VM eggshell hardening. Future identification of other egg activation events in *prg* mutant will help clarify *prg*'s role in *Drosophila* egg activation.

REFERENCES

Ajduk, A., Malagocki, A., and Maleszewski, M. (2008). Cytoplasmic maturation of mammalian oocytes: development of a mechanism responsible for sperm-induced Ca^{2+} oscillations. *Reprod Biol* **8**, 3-22.

Andruss, B.F., Bolduc, C., and Beckingham, K. (2004). Movement of calmodulin between cells in the ovary and embryo of *drosophila*. *Genesis* **38**, 93-103.

Aramburu, J., Garcia-Cozar, F., Raghavan, A., Okamura, H., Rao, A., and Hogan, P.G. (1998). Selective inhibition of NFAT activation by a peptide spanning the calcineurin targeting site of NFAT. *Mol Cell* **1**, 627-637.

Artavanis-Tsakonas, S. (2004). Accessing the Exelixis collection. Nat Genet *36*, 207.

Atkins, C.M., Nozaki, N., Shigeri, Y., and Soderling, T.R. (2004). Cytoplasmic polyadenylation element binding protein-dependent protein synthesis is regulated by calcium/calmodulin-dependent protein kinase II. *J Neurosci* **24**, 5193-5201.

Baker, R.F. (1983). DNA-mediated alteration of the reversion frequency of transformed NIH/3T3 cells. *Proc Natl Acad Sci U S A* **80**, 1174-1178.

Bashirullah, A., Halsell, S.R., Cooperstock, R.L., Kloc, M., Karaiskakis, A., Fisher, W.W., Fu, W., Hamilton, J.K., Etkin, L.D., and Lipshitz, H.D. (1999). Joint action of two RNA degradation pathways controls the timing of maternal transcript elimination at the midblastula transition in *Drosophila melanogaster*. *EMBO J* **18**, 2610-2620.

Benoit, B., Mitou, G., Chartier, A., Temme, C., Zaessinger, S., Wahle, E., Busseau, I., and Simonelig, M. (2005). An essential cytoplasmic function for the nuclear poly(A) binding protein, PABP2, in poly(A) tail length control and early development in *Drosophila*. *Dev Cell* **9**, 511-522.

Berridge, M.J., Bootman, M.D., and Roderick, H.L. (2003). Calcium signalling:

dynamics, homeostasis and remodelling. *Nat Rev Mol Cell Biol* **4**, 517-529.

Bettegowda, A., and Smith, G.W. (2007). Mechanisms of maternal mRNA regulation: implications for mammalian early embryonic development. *Front Biosci* **12**, 3713-3726.

Bonds, M., Sands, J., Poulson, W., Harvey, C., and Von Ohlen, T. (2007). Genetic screen for regulators of ind expression identifies shrew as encoding a novel twisted gastrulation-like protein involved in Dpp signaling. *Dev Dyn* **236**, 3524-3531.

Brent, A.E., MacQueen, A., and Hazelrigg, T. (2000). The *Drosophila wispy* gene is required for RNA localization and other microtubule-based events of meiosis and early embryogenesis. *Genetics* **154**, 1649-1662.

Brunet, A., and Pouyssegur, J. (1997). Mammalian MAP kinase modules: how to transduce specific signals. *Essays Biochem* **32**, 1-16.

Chang, H.Y., Minahan, K., Merriman, J.A., and Jones, K.T. (2009). Calmodulin-dependent protein kinase gamma 3 (CamKIIgamma3) mediates the cell cycle resumption of metaphase II eggs in mouse. *Development* **136**, 4077-4081.

Chen, B., Harms, E., Chu, T., Henrion, G., and Strickland, S. (2000). Completion of meiosis in *Drosophila* oocytes requires transcriptional control by grauzone, a new zinc finger protein. *Development* **127**, 1243-1251.

Chu, T., Henrion, G., Haegeli, V., and Strickland, S. (2001). *Cortex*, a *Drosophila* gene required to complete oocyte meiosis, is a member of the Cdc20/fizzy protein family. *Genesis* **29**, 141-152.

Copeland, P.R., and Wormington, M. (2001). The mechanism and regulation of deadenylation: identification and characterization of *Xenopus* PARN. *RNA* **7**, 875-886.

Cui, J., Sackton, K.L., Horner, V.L., Kumar, K.E., and Wolfner, M.F. (2008). Wispy, the *Drosophila* homolog of GLD-2, is required during oogenesis and egg activation. *Genetics* **178**, 2017-2029.

Dehlin, E., Wormington, M., Korner, C.G., and Wahle, E. (2000). Cap-dependent deadenylation of mRNA. *EMBO J* **19**, 1079-1086.

Doyle, K.E., Kovalick, G.E., Lee, E., and Beckingham, K. (1990). Drosophila melanogaster contains a single calmodulin gene. Further structure and expression studies. *J Mol Biol* **213**, 599-605.

Driever, W., and Nusslein-Volhard, C. (1988). The bicoid protein determines position in the Drosophila embryo in a concentration-dependent manner. *Cell* **54**, 95-104.

Ducibella, T., and Fissore, R. (2008). The roles of Ca^{2+}, downstream protein kinases, and oscillatory signaling in regulating fertilization and the activation of development. *Dev Biol* **315**, 257-279.

Ducibella, T., Huneau, D., Angelichio, E., Xu, Z., Schultz, R.M., Kopf, G.S., Fissore, R., Madoux, S., and Ozil, J.P. (2002). Egg-to-embryo transition is driven by differential responses to $Ca(^{2+})$ oscillation number. *Dev Biol* **250**, 280-291.

Ducibella, T., Schultz, R.M., and Ozil, J.P. (2006). Role of calcium signals in early development. *Semin Cell Dev Biol* **17**, 324-332.

Edgar, B.A., and Datar, S.A. (1996). Zygotic degradation of two maternal Cdc25 mRNAs terminates *Drosophila*'s early cell cycle program. *Genes Dev* **10**, 1966-1977.

Fan, H.Y., and Sun, Q.Y. (2004). Involvement of mitogen-activated protein kinase cascade during oocyte maturation and fertilization in mammals. *Biol Reprod* **70**, 535-547.

Frank, P., Braunshofer-Reiter, C., Karwan, A., Grimm, R., and Wintersberger, U. (1999). Purification of Saccharomyces cerevisiae RNase H(70) and identification of the corresponding gene. *FEBS Lett* **450**, 251-256.

Fuentes, J.J., Genesca, L., Kingsbury, T.J., Cunningham, K.W., Perez-Riba, M., Estivill, X., and de la Luna, S. (2000). DSCR1, overexpressed in Down syndrome, is

an inhibitor of calcineurin-mediated signaling pathways. *Hum Mol Genet* **9**, 1681-1690.

Giraldez, A.J., Mishima, Y., Rihel, J., Grocock, R.J., Van Dongen, S., Inoue, K., Enright, A.J., and Schier, A.F. (2006). Zebrafish MiR-430 promotes deadenylation and clearance of maternal mRNAs. *Science* **312**, 75-79.

Griffith, L.C. (2004). Calcium/calmodulin-dependent protein kinase II: an unforgettable kinase. *J Neurosci* **24**, 8391-8393.

Griffith, L.C., Verselis, L.M., Aitken, K.M., Kyriacou, C.P., Danho, W., and Greenspan, R.J. (1993). Inhibition of calcium/calmodulin-dependent protein kinase in *Drosophila* disrupts behavioral plasticity. *Neuron* **10**, 501-509.

Guan, K.L., and Dixon, J.E. (1991). Eukaryotic proteins expressed in Escherichia coli: an improved thrombin cleavage and purification procedure of fusion proteins with glutathione S-transferase. *Anal Biochem* **192**, 262-267.

Gwack, Y., Feske, S., Srikanth, S., Hogan, P.G., and Rao, A. (2007). Signalling to transcription: store-operated Ca^{2+} entry and NFAT activation in lymphocytes. *Cell Calcium* **42**, 145-156.

Hacker, U., Lin, X., and Perrimon, N. (1997). The *Drosophila* sugarless gene modulates Wingless signaling and encodes an enzyme involved in polysaccharide biosynthesis. *Development* **124**, 3565-3573.

Halet, G., Marangos, P., Fitzharris, G., and Carroll, J. (2003). Ca^{2+} oscillations at fertilization in mammals. *Biochem Soc Trans* **31**, 907-911.

Harms, E., Chu, T., Henrion, G., and Strickland, S. (2000). The only function of Grauzone required for *Drosophila* oocyte meiosis is transcriptional activation of the cortex gene. *Genetics* **155**, 1831-1839.

Heifetz, Y., Yu, J., and Wolfner, M.F. (2001). Ovulation triggers activation of *Drosophila* oocytes. *Dev Biol* **234**, 416-424.

Hilioti, Z., Gallagher, D.A., Low-Nam, S.T., Ramaswamy, P., Gajer, P., Kingsbury,

T.J., Birchwood, C.J., Levchenko, A., and Cunningham, K.W. (2004). GSK-3 kinases enhance calcineurin signaling by phosphorylation of RCNs. *Genes Dev* **18**, 35-47.

Horner, V.L., Czank, A., Jang, J.K., Singh, N., Williams, B.C., Puro, J., Kubli, E., Hanes, S.D., McKim, K.S., Wolfner, M.F., *et al.* (2006). The *Drosophila* calcipressin sarah is required for several aspects of egg activation. *Curr Biol* **16**, 1441-1446.

Horner, V.L., and Wolfner, M.F. (2008a). Mechanical stimulation by osmotic and hydrostatic pressure activates *Drosophila* oocytes in vitro in a calcium-dependent manner. *Dev Biol* **316**, 100-109.

Horner, V.L., and Wolfner, M.F. (2008b). Transitioning from egg to embryo: triggers and mechanisms of egg activation. *Dev Dyn* **237**, 527-544.

Houseley, J., and Tollervey, D. (2009). The many pathways of RNA degradation. *Cell* **136**, 763-776.

Jain, J., McCaffrey, P.G., Miner, Z., Kerppola, T.K., Lambert, J.N., Verdine, G.L., Curran, T., and Rao, A. (1993). The T-cell transcription factor NFATp is a substrate for calcineurin and interacts with Fos and Jun. *Nature* **365**, 352-355.

Johnson, E., Sherry, T., Ringo, J., and Dowse, H. (2002). Modulation of the cardiac pacemaker of *Drosophila*: cellular mechanisms. *J Comp Physiol B* **172**, 227-236.

Jones, K.T. (2005). Mammalian egg activation: from Ca2+ spiking to cell cycle progression. *Reproduction* **130**, 813-823.

Kingsbury, T.J., and Cunningham, K.W. (2000). A conserved family of calcineurin regulators. *Genes Dev* **14**, 1595-1604.

Knott, J.G., Gardner, A.J., Madgwick, S., Jones, K.T., Williams, C.J., and Schultz, R.M. (2006). Calmodulin-dependent protein kinase II triggers mouse egg activation and embryo development in the absence of Ca^{2+} oscillations. *Dev Biol* **296**, 388-395.

Krizanova, O., and Ondrias, K. (2003). The inositol 1,4,5-trisphosphate receptor--

transcriptional regulation and modulation by phosphorylation. *Gen Physiol Biophys* **22**, 295-311.

Kumano, M., Carroll, D.J., Denu, J.M., and Foltz, K.R. (2001). Calcium-mediated inactivation of the MAP kinase pathway in sea urchin eggs at fertilization. *Dev Biol* **236**, 244-257.

Kurokawa, M., Sato, K., and Fissore, R.A. (2004). Mammalian fertilization: from sperm factor to phospholipase Czeta. *Biol Cell* **96**, 37-45.

Kushner, S.R. (2004). mRNA decay in prokaryotes and eukaryotes: different approaches to a similar problem. *IUBMB Life* **56**, 585-594.

Lee, B., Yoon, S.Y., and Fissore, R.A. (2006). Regulation of fertilization-initiated [Ca^{2+}]i oscillations in mammalian eggs: a multi-pronged approach. *Semin Cell Dev Biol* **17**, 274-284.

Lieberfarb, M.E., Chu, T., Wreden, C., Theurkauf, W., Gergen, J.P., and Strickland, S. (1996). Mutations that perturb poly(A)-dependent maternal mRNA activation block the initiation of development. *Development* **122**, 579-588.

Lim, H.W., New, L., Han, J., and Molkentin, J.D. (2001). Calcineurin enhances MAPK phosphatase-1 expression and p38 MAPK inactivation in cardiac myocytes. *J Biol Chem* **276**, 15913-15919.

Lindsay, L.L., Hertzler, P.L., and Clark, W.H., Jr. (1992). Extracellular Mg^{2+} induces an intracellular Ca^{2+} wave during oocyte activation in the marine shrimp *Sicyonia ingentis*. *Dev Biol* **152**, 94-102.

Lisman, J.E. (1985). A mechanism for memory storage insensitive to molecular turnover: a bistable autophosphorylating kinase. *Proc Natl Acad Sci U S A* **82**, 3055-3057.

Liu, J., and Maller, J.L. (2005). Calcium elevation at fertilization coordinates phosphorylation of XErp1/Emi2 by Plx1 and CaMK II to release metaphase arrest by

cytostatic factor. *Curr Biol* **15**, 1458-1468.

Lorca, T., Cruzalegui, F.H., Fesquet, D., Cavadore, J.C., Mery, J., Means, A., and Doree, M. (1993). Calmodulin-dependent protein kinase II mediates inactivation of MPF and CSF upon fertilization of *Xenopus* eggs. *Nature* **366**, 270-273.

MacDonnell, S.M., Weisser-Thomas, J., Kubo, H., Hanscome, M., Liu, Q., Jaleel, N., Berretta, R., Chen, X., Brown, J.H., Sabri, A.K., *et al.* (2009). CaMKII negatively regulates calcineurin-NFAT signaling in cardiac myocytes. *Circ Res* **105**, 316-325.

Markoulaki, S., Matson, S., and Ducibella, T. (2004). Fertilization stimulates long-lasting oscillations of CaMKII activity in mouse eggs. *Dev Biol* **272**, 15-25.

McCarron, J.G., McGeown, J.G., Reardon, S., Ikebe, M., Fay, F.S., and Walsh, J.V., Jr. (1992). Calcium-dependent enhancement of calcium current in smooth muscle by calmodulin-dependent protein kinase II. *Nature* **357**, 74-77.

Mehta, S., Li, H., Hogan, P.G., and Cunningham, K.W. (2009). Domain architecture of the regulators of calcineurin (RCANs) and identification of a divergent RCAN in yeast. *Mol Cell Biol* **29**, 2777-2793.

Meyer, S., Temme, C., and Wahle, E. (2004). Messenger RNA turnover in eukaryotes: pathways and enzymes. *Crit Rev Biochem Mol Biol* **39**, 197-216.

Mishima, Y., Giraldez, A.J., Takeda, Y., Fujiwara, T., Sakamoto, H., Schier, A.F., and Inoue, K. (2006). Differential regulation of germline mRNAs in soma and germ cells by zebrafish miR-430. *Curr Biol* **16**, 2135-2142.

Miyazaki, S. (1988). Inositol 1,4,5-trisphosphate-induced calcium release and guanine nucleotide-binding protein-mediated periodic calcium rises in golden hamster eggs. *J Cell Biol* **106**, 345-353.

Miyazaki, S., Shirakawa, H., Nakada, K., and Honda, Y. (1993). Essential role of the inositol 1,4,5-trisphosphate receptor/Ca^{2+} release channel in Ca^{2+} waves and Ca2+

oscillations at fertilization of mammalian eggs. *Dev Biol* **158**, 62-78.

Mochida, S., and Hunt, T. (2007). Calcineurin is required to release *Xenopus* egg extracts from meiotic M phase. *Nature* **449**, 336-340.

Monsma, S.A., and Wolfner, M.F. (1988). Structure and expression of a *Drosophila* male accessory gland gene whose product resembles a peptide pheromone precursor. *Genes Dev* **2**, 1063-1073.

Nariai, M., Tanaka, T., Okada, T., Shirai, C., Horigome, C., and Mizuta, K. (2005). Synergistic defect in 60S ribosomal subunit assembly caused by a mutation of Rrs1p, a ribosomal protein L11-binding protein, and 3'-extension of 5S rRNA in *Saccharomyces cerevisiae*. *Nucleic Acids Res* **33**, 4553-4562.

Nebreda, A.R., and Hunt, T. (1993). The c-mos proto-oncogene protein kinase turns on and maintains the activity of MAP kinase, but not MPF, in cell-free extracts of *Xenopus* oocytes and eggs. *EMBO J* **12**, 1979-1986.

Nishiyama, T., Yoshizaki, N., Kishimoto, T., and Ohsumi, K. (2007). Transient activation of calcineurin is essential to initiate embryonic development in *Xenopus laevis. Nature* **449**, 341-345.

Page, A.W., and Orr-Weaver, T.L. (1996). The *Drosophila* genes grauzone and cortex are necessary for proper female meiosis. *J Cell Sci* **109** *(Pt 7)*, 1707-1715.

Page, A.W., and Orr-Weaver, T.L. (1997). Activation of the meiotic divisions in *Drosophila* oocytes. *Dev Biol* **183**, 195-207.

Paillard, L., and Osborne, H.B. (2003). East of EDEN was a poly(A) tail. *Biol Cell* **95**, 211-219.

Parks, A.L., Cook, K.R., Belvin, M., Dompe, N.A., Fawcett, R., Huppert, K., Tan, L.R., Winter, C.G., Bogart, K.P., Deal, J.E., *et al.* (2004). Systematic generation of high-resolution deletion coverage of the *Drosophila melanogaster* genome. *Nat Genet* **36**, 288-292.

Perrimon, N., Lanjuin, A., Arnold, C., and Noll, E. (1996). Zygotic lethal mutations with maternal effect phenotypes in *Drosophila melanogaster.* II. Loci on the second and third chromosomes identified by P-element-induced mutations. *Genetics* **144**, 1681-1692.

Pesin, J.A., and Orr-Weaver, T.L. (2007). Developmental role and regulation of cortex, a meiosis-specific anaphase-promoting complex/cyclosome activator. *PLoS Genet* **3**, e202.

Rauh, N.R., Schmidt, A., Bormann, J., Nigg, E.A., and Mayer, T.U. (2005). Calcium triggers exit from meiosis II by targeting the APC/C inhibitor XErp1 for degradation. *Nature* **437**, 1048-1052.

Rorth, P. (1998). Gal4 in the *Drosophila* female germline. *Mech Dev* **78**, 113-118.

Runft, L.L., Jaffe, L.A., and Mehlmann, L.M. (2002). Egg activation at fertilization: where it all begins. *Dev Biol* **245**, 237-254.

Rusnak, F., and Mertz, P. (2000). Calcineurin: form and function. *Physiol Rev* **80**, 1483-1521.

Sackton, K.L., Buehner, N.A., and Wolfner, M.F. (2007). Modulation of MAPK activities during egg activation in *Drosophila*. *Fly (Austin)* **1**, 222-227.

Salles, F.J., Lieberfarb, M.E., Wreden, C., Gergen, J.P., and Strickland, S. (1994). Coordinate initiation of *Drosophila* development by regulated polyadenylation of maternal messenger RNAs. *Science* **266**, 1996-1999.

Salles, F.J., and Stricker, S.A. (1995). Rapid and senstive analysis of mRNA polyadenylation states by PCR. *PCR methods Appl*, 317-321.

Sanna, B., Brandt, E.B., Kaiser, R.A., Pfluger, P., Witt, S.A., Kimball, T.R., van Rooij, E., De Windt, L.J., Rothenberg, M.E., Tschop, M.H., *et al.* (2006). Modulatory calcineurin-interacting proteins 1 and 2 function as calcineurin facilitators *in vivo*. *Proc Natl Acad Sci U S A* **103**, 7327-7332.

Santella, L., De Riso, L., Gragnaniello, G., and Kyozuka, K. (1999). Cortical granule translocation during maturation of starfish oocytes requires cytoskeletal rearrangement triggered by InsP3-mediated Ca^{2+} release. *Exp Cell Res* **248**, 567-574.

Schultz, R.M., and Kopf, G.S. (1995). Molecular basis of mammalian egg activation. *Curr Top Dev Biol* **30**, 21-62.

Semotok, J.L., Cooperstock, R.L., Pinder, B.D., Vari, H.K., Lipshitz, H.D., and Smibert, C.A. (2005). Smaug recruits the CCR4/POP2/NOT deadenylase complex to trigger maternal transcript localization in the early *Drosophila* embryo. *Curr Biol* **15**, 284-294.

Sohaskey, M.L., and Ferrell, J.E., Jr. (1999). Distinct, constitutively active MAPK phosphatases function in *Xenopus* oocytes: implications for p42 MAPK regulation *In vivo*. *Mol Biol Cell* **10**, 3729-3743.

Stapleton, M., Carlson, J., Brokstein, P., Yu, C., Champe, M., George, R., Guarin, H., Kronmiller, B., Pacleb, J., Park, S., *et al.* (2002). A *Drosophila* full-length cDNA resource. *Genome Biol* **3**, RESEARCH0080.

Stricker, S.A. (1999). Comparative biology of calcium signaling during fertilization and egg activation in animals. *Dev Biol* **211**, 157-176.

Sun, Q.Y. (2003). Cellular and molecular mechanisms leading to cortical reaction and polyspermy block in mammalian eggs. *Microsc Res Tech* **61**, 342-348.

Sun, Q.Y., Breitbart, H., and Schatten, H. (1999). Role of the MAPK cascade in mammalian germ cells. *Reprod Fertil Dev* **11**, 443-450.

Surdej, P., and Jacobs-Lorena, M. (1998). Developmental regulation of bicoid mRNA stability is mediated by the first 43 nucleotides of the 3' untranslated region. *Mol Cell Biol* **18**, 2892-2900.

Swan, A., Barcelo, G., and Schupbach, T. (2005). *Drosophila* Cks30A interacts with Cdk1 to target Cyclin A for destruction in the female germline. *Development* **132**,

3669-3678.

Swan, A., and Schupbach, T. (2007). The Cdc20 (Fzy)/Cdh1-related protein, Cort, cooperates with Fzy in cyclin destruction and anaphase progression in meiosis I and II in *Drosophila*. *Development* **134**, 891-899.

Swann, K., Saunders, C.M., Rogers, N.T., and Lai, F.A. (2006). PLCzeta(zeta): a sperm protein that triggers Ca^{2+} oscillations and egg activation in mammals. *Semin Cell Dev Biol* **17**, 264-273.

Tadros, W., Goldman, A.L., Babak, T., Menzies, F., Vardy, L., Orr-Weaver, T., Hughes, T.R., Westwood, J.T., Smibert, C.A., and Lipshitz, H.D. (2007). SMAUG is a major regulator of maternal mRNA destabilization in *Drosophila* and its translation is activated by the PAN GU kinase. *Dev Cell* **12**, 143-155.

Tadros, W., Houston, S.A., Bashirullah, A., Cooperstock, R.L., Semotok, J.L., Reed, B.H., and Lipshitz, H.D. (2003). Regulation of maternal transcript destabilization during egg activation in *Drosophila*. *Genetics* **164**, 989-1001.

Tadros, W., and Lipshitz, H.D. (2005). Setting the stage for development: mRNA translation and stability during oocyte maturation and egg activation in *Drosophila*. *Dev Dyn* **232**, 593-608.

Tadros, W., and Lipshitz, H.D. (2009). The maternal-to-zygotic transition: a play in two acts. *Development* **136**, 3033-3042.

Takeo, S., Hawley, R.S., and Aigaki, T. (2010). Calcineurin and its regulation by Sra/RCAN is required for completion of meiosis in *Drosophila*. *Dev Biol* **344**, 957-967.

Takeo, S., Tsuda, M., Akahori, S., Matsuo, T., and Aigaki, T. (2006). The calcineurin regulator sra plays an essential role in female meiosis in *Drosophila*. *Curr Biol* **16**, 1435-1440.

Tamura, K., Miyata, K., Sugahara, K., Onishi, S., Shuin, T., and Aso, T. (2003). Identification of EloA-BP1, a novel Elongin A binding protein with an exonuclease

homology domain. *Biochem Biophys Res Commun* **309**, 189-195.

Tatone, C., Delle Monache, S., Iorio, R., Caserta, D., Di Cola, M., and Colonna, R. (2002). Possible role for Ca(*2+*) calmodulin-dependent protein kinase II as an effector of the fertilization Ca(*2+*) signal in mouse oocyte activation. *Mol Hum Reprod* **8**, 750-757.

Tatone, C., Iorio, R., Francione, A., Gioia, L., and Colonna, R. (1999). Biochemical and biological effects of KN-93, an inhibitor of calmodulin-dependent protein kinase II, on the initial events of mouse egg activation induced by ethanol. *J Reprod Fertil* **115**, 151-157.

Taylor, S.S., Scott, M.I., and Holland, A.J. (2004). The spindle checkpoint: a quality control mechanism which ensures accurate chromosome segregation. *Chromosome Res* **12**, 599-616.

Tsaadon, A., Eliyahu, E., Shtraizent, N., and Shalgi, R. (2006). When a sperm meets an egg: block to polyspermy. *Mol Cell Endocrinol* **252**, 107-114.

Van Doren, M., Williamson, A.L., and Lehmann, R. (1998). Regulation of zygotic gene expression in *Drosophila* primordial germ cells. *Curr Biol* **8**, 243-246.

Van Hoof, A., Lennertz, P., and Parker, R. (2000). Three conserved members of the RNase D family have unique and overlapping functions in the processing of 5S, 5.8S, U4, U5, RNase MRP and RNase P RNAs in yeast. *EMBO J* **19**, 1357-1365.

VanBerkum, M.F., and Goodman, C.S. (1995). Targeted disruption of Ca($^{2+}$)-calmodulin signaling in *Drosophila* growth cones leads to stalls in axon extension and errors in axon guidance. *Neuron* **14**, 43-56.

VanBerkum, M.F., and Means, A.R. (1991). Three amino acid substitutions in domain I of calmodulin prevent the activation of chicken smooth muscle myosin light chain kinase. *J Biol Chem* **266**, 21488-21495.

Wang, B., Sullivan, K.M., and Beckingham, K. (2003). *Drosophila* calmodulin

mutants with specific defects in the musculature or in the nervous system. *Genetics* **165**, 1255-1268.

Waring, G.L. (2000). Morphogenesis of the eggshell in *Drosophila. Int Rev Cytol* **198**, 67-108.

Weinstein, H., and Mehler, E.L. (1994). $Ca(^{2+})$-binding and structural dynamics in the functions of calmodulin. *Annu Rev Physiol* **56**, 213-236.

Went, D.F., and Krause, G. (1974). Egg activation in *Pimpla turionellae* (Hym.). *Naturwissenschaften* **61**, 407-408.

Wessel, G.M., Brooks, J.M., Green, E., Haley, S., Voronina, E., Wong, J., Zaydfudim, V., and Conner, S. (2001). The biology of cortical granules. *Int Rev Cytol* **209**, 117-206.

White-Cooper, H., Alphey, L., and Glover, D.M. (1993). The cdc25 homologue twine is required for only some aspects of the entry into meiosis in *Drosophila*. *J Cell Sci* **106** *(Pt 4)*, 1035-1044.