FUNCTION AND REGULATION OF MATERNAL PROTEINS THAT ARE PHOSPHOREGULATED DURING EGG ACTIVATION

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FUNCTION AND REGULATION OF MATERNAL PROTEINS THAT ARE PHOSPHOREGULATED DURING EGG ACTIVATION

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Egg activation is the essential transition through which a mature oocyte becomes a developmentally competent egg. During this transition, the oocyte completes meiosis and remodels its transcriptome and proteome through post-transcriptional and post-translational regulations to prepare for embryogenesis. Phosphoregulation is a particularly important type of post-translational modification at the time of egg activation. In addition to the prevalence of protein phosphorylation state changes during this transition, conserved phosphoregulators like calcineurin and CaMKII are essential for egg activation in different species. Since the phosphorylation state of a protein is tightly associated with its activities, the proteins that are subject to phosphoregulation during egg activation are likely involved in the transition from oocyte to embryo.

In this dissertation, I present my studies on the function and regulation of proteins that go through phosphorylation state changes during egg activation in Drosophila. With germline-specific RNAi, I tested the function of 189 such proteins in female fertility, and identified 53 proteins whose germline depletion led to defective oogenesis, as well as 51 proteins whose germline depletion led to significant impairment or abolishment of the eggs' ability to hatch. By carefully examining the knockdown phenotypes, I identified a set of regulators that are essential for processes

in both early oogenesis and early embryogenesis, and revealed 15 proteins with new roles in egg activation and embryogenesis.

To explore the mechanisms that mediate the phosphorylation state changes of proteins during egg activation, I investigated the activity of phosphoregulator calcineurin in this transition using proteomic and phosphoproteomic analysis. I quantified the phosphorylation state changes and protein abundance changes that occur during egg activation, and examined how these changes are affected when calcineurin function is perturbed in female germ cells. I showed that calcineurin is involved in the regulation of hundreds of phosphosites upon egg activation, and is also required for the abundance changes of numerous proteins during this transition. My results indicate that eggs with perturbed calcineurin activity fail to exit metaphase I, likely due to unsuccessful activation of APC/C. I also showed that calcineurin activity is required for Pan Gu kinase activation, and affects the phosphoregulation of several other regulators of protein translation upon egg activation.

In summary, my studies showed the functional importance of proteins that are phosphoregulated upon egg activation, and shed lights on the mechanism of phosphoregulation during this transition.

BIOGRAPHICAL SKETCH

Zijing Zhang is the only daughter of Xiaozhuang Zheng and Yimin Zhang. She was born and raised in Beijing, China. Her interest in biology was triggered by the many species of animals her family kept as pets at home when she was young. This interest was further nurtured when biology formally showed up in her curriculum as a course in middle school. As a teenager, she often found biology textbooks stimulating in the same way as detective novels, and was fascinated by how scientists, working like detectives, used careful observations and elegantly designed experiments to unveil the great schemes of nature. During her undergraduate study at the University of Kentucky, Zijing majored in biotechnology, and explored several different fields of biological research. She worked for 3 years as a technician in the lab of Dr. Robert Miller, overseeing the molecular genetic screen for desirable genotypes in tobacco plants; and she completed her thesis project studying the purine salvage pathway of equine protozoan parasite *Sarcocystis neurona* in the lab of Dr. Daniel Howe. These research experiences gave her a front-row view in two fields quite distinctly characteristic of Kentucky. Eventually, Zijing developed a strong research interest in reproductive biology during her graduate study at Cornell University, where she worked in the lab of Dr. Mariana Wolfner, studying proteins that are phosphoregulated during egg activation in *Drosophila melanogaster*. Her six years at Cornell was full of adventures, both in her professional and personal life. She engaged with the local community through multiple science outreach programs. She also explored the beautiful natural environment and rich wildlife profile in the finger lake region through numerous hiking and birding trips. Through these activities, she made many

friends for life. And very importantly, she met Lu Huang, with whom she may share the journey through the rest of her life.

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I would also like to thank my committee members Ken Kemphues, and John Schimenti. They have provided me with ample advice on how to proceed with my projects at our committee meetings, and have always been very kind and supportive when I needed help.

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I would also like to thank my colleagues in and around Wolfner lab. I'm grateful that I

have the pleasure to work in such a collaborative and friendly environment. And this working environment is key to help me through my graduate study. Especially, I want to thank Amber Krauchunas for setting the foundation for my graduate projects, and being very understanding when I asked her to dig out ancient data and notes.

Finally, I would like to thank all my families and friends for their support. And most importantly, I would like to thank my parents. When all peers around me were burying themselves in problem sets in preparation for the Chinese college entrance exam, they encouraged their fledgling to go overseas to see the world and learn to be independent. And that was the first step that led me to where I am today.

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CHAPTER 1

INTRODUCTION[1]

1.1 Overview[2]

The fully-grown oocyte is arrested in a species-specific stage of meiosis. In rodents and primates, oocytes within ovarian follicles are arrested in prophase of meiosis I until they undergo meiotic maturation in response to the pituitary-derived luteinizing hormone (LH) surge. The LH surge induces resumption and completion of meiosis I, with extrusion of the first polar body containing half of the chromosomes. The oocyte then progresses to metaphase of meiosis II, at which point meiosis is once again arrested. In addition to these cell cycle changes, oocyte maturation is accompanied by alterations in the cytoplasm such as changes in localization of cytoplasmic organelles including mitochondria and endoplasmic reticulum (ER), accumulation of calcium into ER stores, movement of cortical granules to the oocyte periphery, and translation of stored maternal mRNAs to generate proteins required during early embryo development. The mature oocyte is ready to be fertilized, and is now known as an egg. In starfish and Drosophila, the oocytes are arrested in meiosis I prior to fertilization, and complete both meiosis I and II following egg activation, whereas sea urchin eggs complete meiosis prior to spawning and exposure to sperm. Despite these differences in the exact stage of meiotic arrest, egg activation and fertilization initiate development into a new organism containing the appropriate complement of chromosomes.

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[1] This chapter is adapted from a published encyclopedia entry on egg activation. Zhang Z, Wolfner MF, Williams C (2017) Egg Activation. Encyclopedia for Life Sciences.

[2] This section is mainly written by Dr. Carmen Williams

In vertebrates, mollusks and echinoderms, the events of egg activation are triggered by a signal communicated from the sperm to the egg that initiates the transition of the egg into an early embryo. Egg activation in insects is triggered instead by a mechanical signal that is independent of sperm-egg contact. In essentially all animals, egg activation is mediated by a rise in the level of intracellular calcium, though the patterns of alterations in calcium differ dramatically among the species. In most, the calcium rise initiates formation of a block to polyspermy that prevents fertilization by supernumerary sperm. Calcium-induced signals also cause resumption and eventual completion of meiosis by inducing modulation of cell cycle regulatory proteins. The resulting haploid female nucleus and the newly decondensed paternal nucleus form pronuclei that migrate toward each other. The pronuclear envelopes subsequently break down, and maternal and paternal chromosomes align on the metaphase plate of a single spindle. The first mitotic cell division marks the end of the egg activation process. In the following sections, we review the molecular mechanisms underlying the different steps of egg activation, with a focus on mechanisms common to many species.

1.2 [3]Mechanisms of Calcium Release

The importance of calcium as the initial mediator of egg activation was suggested by observations from pioneering embryologists, including Jacques Loeb, working in the late nineteenth and early twentieth centuries (Loeb 1913). Loeb found that artificial activation of unfertilized sea urchin eggs could be achieved by increasing calcium

[3] This section is mainly written by Dr. Carmen Williams

influx. Years later, the central role of calcium in egg activation during normal fertilization was established in several other species by demonstrations that: (1) there is a natural rise in intracellular calcium in the egg at egg activation; (2) egg activation can be prevented by inhibiting the increase in intracellular calcium; and (3) artificially increasing calcium in the egg initiates activation in the absence of sperm, a process known as “parthenogenetic activation”.

Although the exact mechanisms that increase the egg’s intracellular free calcium levels vary, a common element among most animals is that the source of the calcium is its release from ER stores. In mammals, there is substantial evidence that a sperm-specific phospholipase C (PLC), PLCζ, is the primary inducer of calcium release in the egg (Hachem et al 2017, Knott et al 2005, Saunders et al 2002). Sperm-egg fusion causes the release of PLCζ from the sperm head region into the egg cytoplasm. PLCζ acts on phosphoinositide 4,5-bisphosphate to generate the second messenger inositol 3,4,5-trisphosphate (IP3). IP3 then activates the IP3 receptor on ER membranes to cause calcium release from ER stores. Evidence supporting the essential role of the IP3 receptor was provided by experiments in which microinjection of an antibody that blocks IP3 receptor-mediated calcium release inhibited sperm-induced egg activation (Miyazaki et al 1992, Xu et al 1994).

In sea urchins, the binding of IP3 to the IP3 receptor is also important for calcium release from ER stores. However, IP3 is generated via activity of the γ isoform of PLC (PLCγ), which is activated by Src family kinases through phosphorylation upon sperm-egg membrane fusion (Shearer et al 1999, Tosca et al 2012). In addition to the IP3 receptor, several other types of calcium channels are involved in calcium release,

including ryanodine receptor channels that mediate calcium-induced-calcium-release from the ER (Ogawa 1994), and nicotinic acid adenine dinucleotide-dependent two-pore channels that mediate calcium release from acidic organelles, such as lysosomes (Aarhus et al 1995, Galione et al 2010, Patel et al 2010).

This initial calcium release from the ER causes an elevation in egg cytoplasmic free calcium levels that proceeds in a wave across the egg and can last for several minutes. In frogs and echinoderms, there is only one wave. Cytoplasmic calcium is then brought back to its baseline level via the activity of several types of ATP-dependent calcium pumps. Calcium is pumped back into the ER via the activity of sarco-endoplasmic reticulum calcium ATPases. Calcium is extruded from the egg via plasma membrane calcium ATPases. Finally, calcium is taken up into mitochondria via the activity of the mitochondrial uniporter. In mammalian eggs, the continued presence of PLCζ in the egg's cytoplasm leads to additional cycles of IP3-mediated calcium release and reuptake into the ER, and as a consequence, a series of oscillations in cytoplasmic calcium occur. These calcium oscillations persist for several hours following fertilization, and are necessary for inducing particular events in egg activation (Ducibella et al 2002, Satouh et al 2017). However, because calcium is pumped out of the egg to bring its cytoplasmic calcium level back to baseline, for these subsequent calcium oscillations calcium must be taken up from the external environment to replenish the egg's ER calcium stores. In the absence of extracellular calcium, the ER stores diminish and oscillations do not persist (Igusa & Miyazaki 1983, Kline & Kline 1992). In the mouse, this calcium influx is also essential for

successful emission of the second polar body and is mediated in part by the T-type calcium channel, $Ca_V3.2$ (Bernhardt et al 2015, Miao et al 2012).

Calcium levels also rise concomitant with egg activation in Drosophila oocytes; however, this calcium rise is not triggered by fertilization. Instead, it is triggered by mechanical forces that impact the oocyte as it is ovulated from the ovary. A calcium rise initiates at the posterior end of the oocyte, which is the first part to encounter these forces and a new environment (Kaneuchi et al 2015, York-Andersen et al 2015). In vitro experiments indicate that mechanosensitive ion channels, likely in the TRP family, are required for this calcium rise, mediating uptake of calcium from the external environment. Analogous to the situation in other animals, after the initial local calcium rise, a calcium wave moves through the Drosophila oocyte cytoplasm via the action of IP3-mediated release of calcium from internal stores.

1.3 Zinc Sparks

Mammalian eggs release a large amount of zinc into the extracellular space upon egg activation through exocytotic events termed “zinc sparks” (Kim et al 2011). This phenomenon has been observed in species including mouse, pig, macaque and human. Depletion of zinc from oocytes results in the release of meiotic arrest because activity of the cytostatic factor (CSF, described below) component Emi2 requires zinc as a cofactor (Bernhardt et al 2012, Duncan et al 2016, Lee et al 2015). Zinc sparks closely follow the calcium wave *in vivo,* and are thought to be induced by the calcium wave. There is a strong positive correlation between the magnitude of zinc release and the developmental potential of the mouse oocyte (Zhang et al 2016). Thus, the regulation

of zinc levels in the oocyte maybe another mechanism that induces downstream events of egg activation. However, zinc sparks have not been reported thus far in non-mammals

1.4 Meiosis arrest and resumption

Maintenance of meiotic arrest prior to egg activation requires the stabilization of cytoplasmic factors that drive cell cycle progression. M-phase promoting factor (MPF) is an essential driver of the G2/M transition. MPF consists of a catalytic subunit, cyclin-dependent kinase 1 (Cdk1, a.k.a. Cdc2) and a regulatory subunit, cyclin B. MPF activity peaks at metaphase of both mitotic and meiotic cell cycles, and mediates multiple aspects of cell division including nuclear envelope breakdown and spindle assembly. Exit from M-phase can be triggered by the activity of the Cdc20-activated E3 ubiquitin ligase anaphase-promoting complex/cyclosome (APC/C^{cdc20}). It targets cyclin B for destruction and thus inactivates MPF, thereby allowing meiosis to proceed from metaphase to anaphase.

Meiosis arrest and resumption in vertebrates

Maintenance of metaphase II arrest in vertebrate oocytes relies on the stabilization of MPF, and inhibition of APC/C^{cdc20} by CSF (see Fig. 3 for a summary of conserved features). Xenopus CSF has multiple molecular components including the proto-oncogene MOS, its downstream kinase cascade MEK/MAPK/$p90^{RSK}$, and early mitotic inhibitor 2 (Emi2, a.k.a. XErp1). At metaphase II, the Mos/MEK/MAPK/$p90^{RSK}$ cascade stabilizes Emi2 (Inoue et al 2007, Nishiyama et al 2007), inhibiting APC/C^{cdc20}, activation by binding to Cdc20 and preventing cyclin B

degradation. Spindle assembly checkpoint (SAC) proteins also contribute to establishment and maintenance of the arrest in Xenopus by preventing association of APC/C with Cdc20 (Fang et al 1998, Li et al 1997, Tunquist et al 2003).

The mechanism of CSF arrest in mouse differs in some details from that in Xenopus, as Mos is essential for maintenance but not establishment of the arrest (Colledge et al 1994, Hashimoto et al 1994), and $P90^{RSK}$ and SAC are dispensable for the arrest (Dumont et al 2005, Tsurumi et al 2004). In addition, experimental evidence in mouse indicates that stabilization of MPF requires dynamic phosphorylation of CDK1 by CDC25A and CDC25B to counteract the inhibitory phosphorylation by WEE1B during metaphase II arrest (Kang et al 2013, Oh et al 2013).

Upon egg activation, the calcium wave signals the release of metaphase II arrest by inducing the degradation of EMI2 and consequent activation of APC/C and inactivation of MPF. In Xenopus, the calcium rise activates calmodulin-dependent kinase II (CaMKII) and transiently activates the calcium/calmodulin dependent phosphatase calcineurin (CaN) (Mochida & Hunt 2007, Morin et al 1994). CaMKII primes EMI2 for phosphorylation by Polo-like Kinase 1 (Plx1), which then targets EMI2 to be marked for destruction by SCF-ubiquitin ligase. Transient activity of CaN dephosphorylates both CDC20 and the APC/C component APC3 and likely, thereby, facilitating the activation of APC/C^{Cdc20}.

In mammals, CaMKII is also thought to mediate EMI2 degradation, while the involvement of CaN has not been demonstrated. In mouse, CaMKIIγ is the isoform critical for egg activation (Backs et al 2010). In addition to EMI2 degradation,

CaMKIIγ activates the protein tyrosine kinase WEE1B, which contributes to the resumption of meiosis by inhibiting CDK1 through phosphorylation.

Meiosis arrest and resumption in Drosophila

In *Drosophila melanogaster*, maintenance of metaphase I arrest relies on the tension on meiotic spindles created by opposing forces between spindle poles and kinetochores of chromosomes with crossovers or heterochromatin-mediated achiasmate homologs association (McKim et al 1993, Hughes et al 2009), as well as on the stabilization of MPF. SAC activity is required for the regulation of proper biorientation of the chromosomes and spindle-kinetochore interactions in Drosophila meiosis. Mos is not required required for the establishment or maintenance of meiotic arrest in Drosophila.

Activation of APC/C and inactivation of MPF are required for anaphase I onset in Drosophila oocytes, as in vertebrates. Two Cdc20 related oocyte proteins, Cortex (Cort) and Fizzy (Fzy), are capable of activating the oocyte-specific form of APC/C. APC/C^{Fzy} and APC/C^{Cort} appear to function cooperatively and play partially redundant roles in the exit from meiosis I. However, their functions are distinct from one another during the transition from metaphase II to anaphase II (Pesin & Orr-Weaver 2007, Swan & Schupbach 2007).

CaN is required for aspects of Drosophila egg activation, but the role of CaMKII in egg activation is still unknown. Depletion of the calcineurin catalytic subunit CanB2 in Drosophila oocytes causes anaphase I arrest shortly after the egg activation-induced release of metaphase I arrest (Takeo et al 2010). Sarah (Sra), a calcium dependent regulator of calcineurin, is implicated in the inactivation of MPF upon egg activation:

the majority of *sra* depleted eggs arrest at anaphase I with high levels of Cyclin B-Cdk1 activity (Horner et al 2006, Takeo et al 2010, Takeo et al 2006). The Shaggy/glycogen synthase kinase 3β (GSK3β) regulates Sra through phosphorylation, and is also required for the completion of meiosis (Takeo et al 2012).

1.5 Blocks to Polyspermy[4]

It is important to limit the number of sperm that fuse with the egg, to avoid aneuploidy. The "block to polyspermy" is a name for the mechanisms by which eggs prevent more than the appropriate number of sperm (usually one) from entering the egg following fertilization. Blocks to polyspermy range in nature from electrical changes in the plasma membrane, to formation of a "fertilization envelope", to "hardening" of the zona pellucida, the extracellular matrix surrounding the mammalian egg. The latter two changes are induced in response to the exocytosis of cortical granules that contain enzymes that modify the egg's extracellular coat.

In some species, one of the early consequences of the calcium rise is the opening of calcium-gated ion channels in the membrane. The consequent change in membrane potential (known as the fertilization potential) prevents additional sperm from fusing with the plasma membrane (Jaffe 1976). This rapid response at the level of membrane potential, termed the "fast" or "electrical" block to polyspermy, is important in aquatic animals such as sea urchins and frogs, whose eggs are exposed to millions of sperm almost simultaneously. In many species including mammals, a very early event of egg activation is the calcium-stimulated fusion of cortical granules with the egg plasma

[4] This section is mainly written by Dr. Carmen Williams

membrane. These granules release their contents, which include enzymes that modify the extracellular coat around the egg cell to prevent the entry of supernumerary sperm, creating a mechanical block to polyspermy. In the mouse, the critical enzyme released from the cortical granules is ovastacin, a zinc-dependent metalloendoproteinase (Burkart et al 2012). Ovastacin release causes cleavage of one of the zona pellucida proteins, ZP2. This cleavage event alters the structure of the zona pellucida so that additional sperm can no longer bind and penetrate the zona.

Another block to polyspermy can occur at the level of the plasma membrane. This "membrane block" serves to prevent sperm-egg fusion in cases where extra sperm manage to penetrate the extracellular egg coat prior to establishment of the zona pellucida block to polyspermy. In the mouse, the membrane block to polyspermy may result from loss of a key plasma membrane protein, JUNO, that serves as the egg surface recognition protein for the sperm (Bianchi et al 2014). Some species utilize both fast and slow blocks to polyspermy, whereas others, including mammals, have no fast block and rely only on the more slowly established mechanical and membrane blocks to polyspermy.

Mechanisms for blocking polyspermy in insects are not yet understood. Insect sperm enter the egg through the micropyle, a channel in the egg's chorion. In Drosophila, it has been suggested that the sperm's very long tail protrudes into the micropyle, preventing entry by other sperm, but other chemical or mechanical blocks to polyspermy have not yet been ruled out.

1.6 Regulation of maternal mRNAs

During oogenesis, the oocyte is provisioned with many macromolecules, including maternal mRNAs. Many maternally-deposited mRNAs are essential for driving early development, because the initial stages of embryogenesis occur without significant transcription of the zygotic genome. Translation of maternal transcripts is highly regulated so that their protein products are synthesized at the appropriate time. Polysome analyses in mouse (Potireddy et al 2006) and Drosophila (Eichhorn et al 2016) show significant increases of maternal mRNA translation upon egg activation and some proteins (i.e. Drosophila Bicoid) are only synthesized at this time (Benoit et al 2008, Cui et al 2008).

This translational control of maternal mRNAs is achieved in part through regulated polyadenylation. In mature oocytes, many maternal mRNAs are stored in the cytoplasm in a translationally inactive state with shortened poly-A tails at their 3' ends. These mRNAs are translated after their poly-A tails are elongated upon egg activation. Vertebrate maternal mRNAs are marked for cytoplasmic polyadenylation upon egg activation by consensus 3' cis-regulatory elements termed cytoplasmic polyadenylation elements (CPE), such as stretches of uridine- or cytidine-rich sequence in Xenopus (Paillard et al 2000). CPEs recruit cytoplasmic polyadenylation specificity factors (CPSF), which, in turn, recruit poly-A polymerases to the mRNA. In the mouse, CPEB1 (cytoplasmic polyadenylation element binding protein 1) binds to CPEs in the mRNA encoding DAZL (deleted in azospermia-like), causing its polyadenylation and an increase in its translation (Chen et al 2011). The resulting DAZL protein binds "DAZL elements" in the 3' ends of additional mRNAs and in

turn increases their polyadenylation and translation. The identities of other vertebrate CPSFs that mediate polyadenylation of maternal mRNAs upon egg activation are still unknown.

Although vertebrate CPEs can mediate polyadenylation of mRNAs in Drosophila, these elements are not required for polyadenylation and translation of crucial maternal transcripts, such as *Toll* and *Bicoid*. Instead, a 3' proximal region, the "polyadenylation region" (PR), is essential for targeting mRNAs for egg activation-induced polyadenylation (Coll et al 2010). Polyadenylation of many important maternal transcripts is catalyzed by the Drosophila cytoplasmic poly-A polymerase Wispy (a member of the conserved GLD-2 family; (Benoit et al 2008, Cui et al 2008), but no consensus sequence element has been identified among Wispy targets (Cui et al 2008, Cui et al 2013). Recent evidence suggests the existence of poly-A tail length-independent translational regulation mechanisms in Drosophila because the translation efficiency of many maternal mRNAs is upregulated significantly upon egg activation even when cytoplasmic polyadenylation is disrupted (Eichhorn et al 2016).

Maternal mRNA translation in Drosophila is also regulated by Pan Gu Kinase (PNG). PNG is activated upon egg activation through dephosphorylation of its regulatory subunit Giant Nuclei (Gnu). PNG regulates translation of mRNAs (Eichhorn et al 2016, Kronja et al 2014), including translation repressor Smaug (Smg), which is crucial for later degradation of some maternal mRNAs. Finally, CaN is likely important in translational regulation of Drosophila maternal mRNAs because *sra* mutants show abnormal translation of Smg and Bicoid.

Some maternal mRNAs whose products are no longer required in early embryos are targeted for translational repression and degradation upon egg activation. This degradation process is critical for the maternal to zygotic transition of developmental control. De-adenylation is a major mechanism that mediates the translational repression and destabilization of mRNAs. In Xenopus, embryo deadenylation elements (EDEN) and AUUUA repeats are 2 cis-elements that target maternal transcripts for deadenylation (Paillard et al 1998, Voeltz & Steitz 1998), EDEN can recruit CUG-BP Elav-like family 1 (CLEF1), which mediates multiple aspects of RNA processing including deadenylation (Paillard et al 2003). In Drosophila, Smg mediates the elimination of maternal transcripts by binding to mRNAs that carry Smg response elements (SRE) and recruiting CCR4/POP2/NOT deadenylase complex (Dahanukar et al 1999, Tadros et al 2007). Recently, a predicted Drosophila exonuclease Prage has been identified as a possible component of the machinery that degrades maternal mRNAs (Cui et al 2016, Tadros et al 2003). It is unclear whether Prage and Smg function independently, or are part of the same pathway. In the mouse, oocyte maturation triggers phosphorylation of the major mRNA-binding protein, MSY2, causing instability of endogenous mRNAs (Medvedev et al 2008). Two mRNA decapping enzymes, DCP1 and DCP2, and two components of the CCR4-NOT deadenylation complex, CNOT6l and CNOT7, are recruited for translation during oocyte maturation (Ma et al 2015). These proteins play essential roles in the degradation of maternal mRNAs. An additional mechanism that destroys some maternal mRNAs is mediated by small non-coding RNAs. In Drosophila, piRNAs are involved in the destabilization of certain mRNAs (Barckmann et al 2015,

Rouget et al 2010). Sometime after egg activation, but still in early development in some organisms, zygotic transcription produces microRNAs that also are important for degradation of particular maternal mRNAs (e.g. in Drosophila and zebrafish; Bushati et al 2008, Giraldez et al 2006).

1.7 Regulation of maternal proteins

Maternally deposited proteins are also dynamically regulated upon egg activation. For example, in Xenopus and mouse, the degradation of cyclin B1 and securin, mediated in part by APC/C, is essential for the release of meiotic arrest. Post-translational modifications of proteins are also modulated during egg activation, providing a means to regulate the activity or stability of these proteins in the absence of new transcription. For example, in vertebrates, several MPF and CSF components are regulated through phosphorylation/dephosphorylation during egg activation, and this is crucial for the resumption of meiosis. In Drosophila, the dephosphorylation of Gnu and Young arrest (YA) are required for the onset of embryonic mitosis (Renault et al 2003, Sackton et al 2009).

These specific examples, and the fact that both CaMKII and CaN modulate protein phosphorylation suggest that phosphoregulation might be a major way by which the activity or stability of critical proteins are regulated during egg activation. Consistent with this hypothesis, protein phosphorylation and dephosphorylation changes are prevalent in sea urchin, and Drosophila during this transition (Guo et al 2015, Krauchunas et al 2012). For example, in sea urchin there is a 23% decrease in the number of detectable proteins in the oocyte in the first 2 min after fertilization, likely

due to degradation and post translational modifications (Roux et al 2006). Global modulation of the phopshoproteome could provide a way to rapidly and coordinately change the activity of proteins that had promoted oocyte maturation (on the one hand) and egg activation (on the other).

1.8 Formation of the male pronucleus

Upon fertilization, the sperm nucleus is extensively modified by components in the egg cytoplasm, thereby forming a male pronucleus. Sperm chromatin is tightly packaged by association with nuclear basic proteins, particularly protamines that are highly crosslinked by disulfide bonds. The egg's cytoplasm is rich in glutathione, which generates a strong reducing environment that breaks these disulfide bonds to initiate decondensation of the sperm head (Perreault et al 1984). Sperm-specific nuclear proteins are rapidly removed from the sperm chromatin and replaced by maternal histone variants. In Xenopus and mouse, the histone chaperone nucleoplasmin (Npm) plays a crucial role in both the removal of protamine and deposition of maternal histones H2A and H2B onto the sperm chromatin (Dingwall & Laskey 1990, Philpott et al 1991). In Xenopus, another histone chaperone, N1/N2, is thought to be involved in the deposition of maternal histones H3 and H4 onto sperm chromatin (Kleinschmidt et al 1986). Incorporation of maternal histone variant H3.3 is also crucial for the decondensation of sperm nucleus. Histone chaperone HIRA mediates the deposition of maternal H3.3 onto sperm chromatin in several species including Drosophila, Xenopus and mouse (Lin et al 2014, Loppin et al 2005, Ray-Gallet et al 2002). Together, these changes reorganize the sperm chromatin into more

standard nucleosome-based chromatin. Establishment of the DNA-histone nucleosome structure in the egg cytoplasm results in recruitment of nuclear lamins, nucleoporins, ER membranes and other components to form a nuclear envelope around the paternal chromatin. Microtubules become associated with nuclear envelope proteins, and along with dynein and dynactin mediate the movement of the male and female pronuclei to an apposed position within the newly formed zygote (Payne et al 2003). Reorganization of the sperm nucleus is likely driven by the calcium increase in the activating egg. In Xenopus, inhibition of the calcium wave blocks formation of the male pronucleus (Kline 1988). And in Drosophila, the calcium/calmodulin dependent calcineurin regulator, Sra, is crucial for the completion of sperm chromatin decondensation and subsequent DNA replication (Horner et al 2006).

1.9 Summary[5]

Egg activation is the essential process by which a mature oocyte – a terminally-differentiated, arrested cell – transitions to being able to undertake early embryogenesis as the totipotent zygote. Induced by a rise in free-calcium levels in the oocyte cytoplasm, egg activation includes processes such as cell cycle regulation (resumption and completion of meiosis), modification of the oocyte's plasma membrane and/or envelopes to prevent polyspermy, and regulation of the stability and translation of RNAs that were stored in the oocyte. In vertebrates and echinoderms, fertilization triggers the critical calcium rise. Molecules delivered by the sperm induce release of calcium from internal stores, causing a wave of increased calcium to

[5] This section is mainly written by Dr. Mariana Wolfner

traverse the egg; in mammals, additional calcium-level oscillations occur due to uptake of calcium from the environment, and in mammals at least there is also release of zinc from the oocyte ("zinc sparks"). In Drosophila, a calcium rise and wave also occur, but these are independent of fertilization and are caused instead by mechanical forces that the oocyte experiences as it leaves the ovary; these forces allow calcium entry through mechanically sensitive ion channels. Despite these differences in induction of the calcium wave, its transit and the macromolecular and cellular events that it triggers are remarkably similar across species, and are carried out by highly conserved regulatory cascades (varying in some taxon-specific details). Future studies to determine the molecular signaling cascades important for egg activation events in different species will provide candidate targets for clinical therapeutic interventions for humans, agricultural animals and endangered species, as well as opportunities to improve reproductive controls in insects.

Dissertation Outline

My graduate studies mainly focused on two aspects of protein phosphoregulation upon egg activation: the phosphoregulated proteins and phosphoregulators.

In Chapter Two of this dissertation, I screened through 189 genes whose protein products alter phosphorylation state during egg activation. In this screen, I identified new factors that are essential for the development of oocyte and early embryos. By characterizing the knockdown phenotypes, I provided clues on the potential functions of these genes at these developmental times.

In the second part of this dissertation, I investigated the activity of phosphatase calcineurin during egg activation by identifying the misregulations of

phosphoproteomic and global proteomic changes during egg activation caused by the depletion of calcineurin regulatory subunit CanB2 in female germ cells. I found that calcineurin is essential for the proper phosphoregulation of hundreds of phosphorylation sites during egg activation. I also found that the perturbation of calcineurin activity caused elevated Cdk1 activity, failure of APC/C activation and abnormal protein translation in activated eggs.

In Appendix A, I tested the efficiency of a new CRISPR/Cas9-mediated germline-specific knockout system using three genes that are known to be required for gametogenesis or early embryo development. My results provided insights for our collaborated efforts with Han lab to develop CRISPR/Cas9 mediated germline-specific knockout as a powerful tool to study gene functions in germ cells.

In Appendix B, I generated a deletion of gene *mrityu* using CRISPR/Cas9 mediated mutagenesis and assessed the fertility of *mri* knockout females.

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CHAPTER 2

MATERNAL PROTEINS THAT ARE PHOSPHO-REGULATED UPON EGG ACTIVATION INCLUDE CRUCIAL FACTORS FOR OOGENESIS, EGG ACTIVATION AND EMBRYOGENESIS IN *DROSOPHILA MELANOGASTER*

2.1 Abstract

Egg activation is essential for the successful transition from a mature oocyte to a developmentally competent egg. It consists of a series of events including the resumption and completion of meiosis, initiation of translation of some maternal mRNAs and destruction of others, and changes to the vitelline envelope. This major change of cell state is accompanied by large scale alteration in the oocyte's phosphoproteome. We hypothesize that the cohort of proteins that are subject to phosphoregulation during egg activation are functionally important for processes before, during, or soon after this transition, potentially uniquely or as proteins needed for essential cellular functions in other (somatic) cells. In this study, we used germline-specific RNAi to examine the function of 189 maternal proteins that are phosphoregulated during egg activation in Drosophila melanogaster. We identified 53 genes whose knockdown reduced or abolished egg production and caused a range of defects in ovarian morphology, as well as 51 genes whose knockdown led to significant impairment or abolishment of the egg hatchability. We observed different stages of developmental arrest in the embryos and various defects in spindle morphology and aberrant centrosome activities in the early arrested embryos. Our results, validated by the detection of multiple genes with previously-documented

maternal effect phenotypes among the proteins we tested, revealed 15 genes with newly discovered roles in egg activation and early embryogenesis in Drosophila. Given that protein phosphoregulation is a conserved characteristic of this developmental transition, we suggest that the phosphoregulated proteins may provide a rich pool of candidates for the identification of important players in the egg-to-embryo transition.

2.2 Introduction

At the end of oogenesis, mature oocytes arrest in meiosis with repressed transcription and a rich deposit of maternal mRNAs and proteins. For development to proceed, the oocyte needs to restart some quiescent cellular activities, and end other ongoing activities. This transition occurs through the process of egg activation, which consists of a series of cellular events including the resumption and completion of meiosis, modifications of the outer egg coverings, and dynamic regulation of maternal mRNAs (Horner & Wolfner 2008b, Krauchunas & Wolfner 2013, Laver et al 2015, Machaty et al 2017, Marcello & Singson 2010, Von Stetina & Orr-Weaver 2011). Egg activation is an intricate process through which the oocyte shifts gears from oogenesis to embryogenesis, from a differentiated gamete into a totipotent zygote that gives rise to all the cell types of the adult. Some of the regulatory molecules for this essential developmental transition have been identified genetically (e.g. in Drosophila (Cui et al 2016, Cui et al 2008, Elfring et al 1997, Freeman et al 1986, Lee et al 2003, Lim et al 2016, Lin & Wolfner 1991, Renault et al 2003, Tadros et al 2007), but the number is

surprisingly small. It seems likely that many molecules essential for this transition cannot be identified via traditional maternal
effect screens due to their essentiality for the organism's survival.

Egg activation initiates with a rise of Ca^{2+} level in the cytoplasm of the oocyte. This calcium rise is triggered by fertilization in vertebrates and some invertebrates (Knott et al 2005, Saunders et al 2002, Singaravelu & Singson 2013, Steinhardt et al 1977, Yoon et al 2012). In insects, the calcium rise is triggered by the passage of the oocyte through the reproductive tract, and is induced independent of fertilization (Horner & Wolfner 2008a, Kaneuchi et al 2015, Sartain & Wolfner 2013, York-Andersen et al 2015). Since significant transcription does not occur during oocyte maturation and egg activation (De Renzis et al 2007, Newport & Kirschner 1982, Zalokar 1976), the dowry of maternal mRNAs and proteins deposited into the oocyte must include all the essential machinery to regulate the cellular events of egg activation in response to the calcium rise. Furthermore, a study in Drosophila found that meiosis can be completed even when translation is inhibited (Page & Orr-Weaver 1997). These findings suggest that the regulation of egg activation, and the early embryonic events that immediately follow, relies heavily on maternally-provided proteins. However, some of these proteins might need to be active at one stage in this transition (e.g. oogenesis) and inactive at another (e.g. early embryogenesis) or the reverse, and thus might be post-translationally modulated to accomplish this.

During egg activation, the oocyte proteome is dynamically regulated through degradation and post-translational modification (Guo et al 2015, Krauchunas et al 2012, Kronja et al 2014a, Laver et al 2015, Presler et al 2017, Roux et al 2006).

Among the latter processes, changes in protein phosphorylation state are very prevalent during this transition. In *Drosophila melanogaster*, over 300 maternal proteins alter their phosphorylation state during egg activation (Krauchunas et al 2012), including a few previously shown to be crucial for egg activation and the onset of syncytial divisions, such as Young arrest (YA) and Giant nuclei (GNU) (Lin & Wolfner 1991, Renault et al 2003, Sackton et al 2009, Yu et al 1999). In sea urchins, changes at hundreds of phosphosites were reported to occur within 2 min and 5 min post fertilization (Guo et al 2015, Roux et al 2006), and in Xenopus ~500 phosphosites show dynamic regulation following fertilization (Presler et al 2017). Consistent with the remodeling of oocyte phosphoproteome seen during the egg-to-embryo transition, two highly-conserved calcium-dependent regulators that are essential for egg activation in several species, Ca2+/Calmodulin dependent kinase II (CaMKII) (Xenopus and mouse (Backs et al 2010, Morin et al 1994)) and calcineurin (Xenopus and Drosophila; (Horner et al 2006, Mochida & Hunt 2007, Takeo et al 2010, Takeo et al 2006)), are a kinase and a phosphatase respectively (Hudmon & Schulman 2002, Rusnak & Mertz 2000). These findings suggest that phosphoregulation is a conserved, functionally important regulatory mechanism that modulates protein activities upon egg activation. Thus, the set of proteins that change in phosphorylation state at this time may be an excellent candidate pool for identification of factors involved in processes before, during and after egg activation, with phosphorylation being a rapid way to turn on/off their activities at the appropriate time. Such molecules could include ones specific to the female germline or the early embryo, or proteins needed

commonly for cellular events later in development (but not yet tested for roles in the germline or egg activation).

Consistent with this hypothesis, 174 conserved proteins alter their phosphorylation states during the egg-to-embryo transition in both sea urchin and the Drosophila (Krauchunas et al 2012, Guo et al 2015), suggesting that a cohort of phosphoregulated proteins is deeply conserved, and may be functionally important for the transition from oocyte to embryo.

Here, we tested 189 proteins that are phosphoregulated upon egg activation in *Drosophila melanogaster* (Krauchunas et al 2012) for their involvement in oocyte formation and/or the transition from oocyte to early embryo. The UAS-Gal4 system (Brand & Perrimon 1993) enabled us to dissect the function of our target genes by robust germline-specific RNAi knockdown of their expression (Ni et al 2011), thus avoiding the lethality that could have arisen in a whole-organism mutational screen. We identified 53 genes whose products are crucial for oogenesis. We also observed maternal effect phenotypes upon knockdown of 51 genes, 27 of which caused significant arrest in early embryogenesis, indicating vital roles of their products in the initiation of zygotic development. These genes encode factors known to be involved in various cellular processes in somatic cells, such as mitosis and metabolism. Twelve of these 27 genes have previously been reported to have maternal effects, thus validating our screen and indicating that the other 15 genes are likely new regulators of this process. We also show that many factors involved in mitosis and other processes in later somatic cells are also essential for the development of oocyte and the syncytial divisions in early embryos. Our study establishes the cohort of maternal proteins that

are phosphoregulated during egg activation as a rich candidate pool for the identification of factors essential for female fertility.

2.3 Materials and methods

Fly stocks: Fly stocks were maintained on standard yeast-glucose-agar media, at 23±2°C, under a 12-hr light-dark cycle.

Germline specific RNAi: A total of 207 transgenic shRNA lines (Ni et al 2011, Perkins et al 2015) carrying -WALIUM or VALIUM- vectors driving shRNA targeting 189 candidates were obtained from the Bloomington stock center, the NIG stock center, and the Vienna Drosophila Resource Center (stock numbers are listed in Table S2.1). 5-10 virgin females from the shRNA stock were mated to 5-10 males carrying either MTD-Gal4, which drives expression throughout oogenesis (Petrella et al 2007), or matα4-GAL-VP16, which drives expression after the germarium stages (Radford et al 2012, Sugimura & Lilly 2006). These crosses generated females that are heterozygous for both shRNA and the driver construct (referred to here as "RNAi females"). Background-matched control females, heterozygous for the AttP2 (empty insertion site of the shRNA construct) and the appropriate driver construct were generated in parallel to serve as controls. RNAi females and control females were both raised and maintained at 27°C.

Fertility assays: Virgin RNAi females and control females were mated to Oregon R P2 (Allis et al 1977) males in single pairs; mating was confirmed by observation. Males were removed after mating and females were allowed to lay eggs on standard fly media and were transferred to new culture vials every 24 hours for 4 days. The

number of eggs laid in each vial was counted. The hatch rate was estimated as the proportion of eggs that developed into pupae. For each assay, RNAi females for 5-10 TRiP lines (5-10 RNAi females for each line) and 1 control group (10 control females) were included. Total 4-day egg production and overall embryo hatchability were calculated for each female. The 4-day egg production and embryo hatchability of the 5-10 RNAi females for each TRiP line were compared to those of the 10 control females from the same assay using Student's T-test (Table S2.1). RNAi lines that generated fertility phenotypes were re-tested in 2-3 independent biological replicates, to ensure reproducibility of the results.

*Reverse-transcription PCR***:** S Stage 14 oocytes were dissected in hypertonic Isolation Buffer, which prevents egg activation (Page & Orr-Weaver 1997). RNA was extracted using TRIzol® (Thermo Fisher). RNA samples were treated with DNase (Promega) and reverse transcribed with SmartScribe Reverse Transcriptase Kits (CloneTech Laboratories, Inc). PCR was performed with cDNA samples using primers for the gene being tested for knockdown and primers for Rpl32 as control; primers are listed in Table S2.2. Band intensities of the amplicons from knockdown and control samples were quantified and compared using ImageJ, as described in Findlay et al 2014 (Findlay et al 2014). For each sample tested (knockdown, control) amplicon band intensity of the target gene was normalized to that of Rpl32. We focused our analysis on the knockdowns that gave 40% or less of control expression of the targeted gene (Sopko et al 2014).

Bioinformatics: Peptide sequences were scanned for consensus phosphorylation site using Scansite 2.0 Motif Scan (Obenauer et al 2003). The result included consensus phosphorylation sites identified on all the isoforms of each gene.

Immunofluorescence: 2-4hr old and 0.5-1.5hr old fertilized embryos were collected on grape juice agar plates, de-chorionated with 50% bleach, fixed using heptane/methanol and stained as described in (Horner et al 2006). Ovaries from 3-5-day old females were dissected in Isolation Buffer (Page & Orr-Weaver 1997) and fixed using Fixation Buffer (Radford & McKim 2016), incubated with 5µg/ml RNaseA overnight (Horner et al 2006), and stained with propidium iodide. Mouse anti-αtubulin (Sigma, St. Louis, MO, catalog #T5168) was used at a dilution of 1:400. Mouse anti-γtubulin (Boster Biological Technologies, Pleasanton, CA, catalog #MA1114) was reconstituted in 1ml of PBS and used at a dilution of 1:100. Mouse anti-DROP-1, which stains sperm tails (Karr 1991), (kindly provided by T. Karr at Kyoto University) was used at 1:800 as described in Krauchunas et al 2012. Alexa Fluor® 488 conjugated anti-mouse (Thermo Fisher) was used at 1:200. Propidium iodide was used at 10 µg/ml. Samples were examined and images were generated using a Leica TCS SP2 confocal microscope at the Cornell Imaging Core.

Immunoblotting: 80-100 oocytes or activated but unfertilized eggs (collected from females mated to the spermless sons of *tudor* females) (Boswell & Mahowald 1985) were homogenized in 10 µl of Protein Extraction Buffer (10 mM Tris, pH 7.5; 20 mM NaF, 2 mM EGTA, 10 mM DTT, 400 nM okadaic acid, and 2% SDS), and the lysate was mixed with 10 µl of SDS loading buffer. Proteins were separated by electrophoresis in 8% polyacrylamide SDS gels. Primary guinea pig anti-Smg

antibody (Semotok et al 2005) (kindly provided by H. Lipshitz at University of Toronto) was used at 1:10000. Mouse anti-tubulin was used at dilution of 1:10000 (Sigma, St. Louis, MO, catalog #T5168). Secondary HRP conjugated anti-guinea pig was used at 1:1000 (Jackson Laboratories). Anti-mouse secondary antibody was used at 1:2000 (Jackson Laboratories).

2.4 Results

Germline specific depletion of phosphoregulated maternal proteins reveals factors crucial for different aspects of female fertility.

To test whether the subset of proteins that are phosphoregulated during egg activation are important for female fertility in *Drosophila melanogaster*, we obtained all available TRiP RNAi lines that targeted the phosphoregulated maternal proteins identified by Krauchunas et al 2012 (Krauchunas et al 2012).

A total of 207 TRiP lines targeting 189 genes were obtained. Utilizing the proteomic data published in a recent study of posttranscriptional changes during Drosophila egg activation (Kronja et al 2014a), we examined the abundance changes of the proteins we studied. Of the 126 proteins with proteomic data available, the abundance of 112 did not change significantly during the transition from oocyte to embryo (Table S2.1). This supports our view that phosphoregulation is the mechanism that modulates these proteins at this transition.

In our primary screen, all 207 TRiP lines, were screened using the MTD-Gal4 driver which drives shRNA production throughout oogenesis (Petrella et al 2007). For the genes whose knockdown led to severely reduced or abolished egg production, we

performed a secondary screen using matα4-GAL-VP16, which drives shRNA expression in mid and late oogenesis (Radford et al 2012, Sugimura & Lilly 2006). We expected that for genes that have crucial functions in early oogenesis, the temporal manipulation of RNAi knockdown could allow the depletion of gene products in mature oocytes without severely impacting egg production. We grouped the 189 genes into six classes according to their knockdown phenotypes in the primary and the secondary screen (Table 2.1, Figure 1A, Table S2.1).

The 81 genes in Classes 2 to 6 whose knockdown caused a range of defects in the female germline or early embryos will be discussed in more detail below. For the remaining 108 genes (Class 1), knockdown of their expression throughout oogenesis (MTD-Gal4>UAS-shRNA) did not impact female fertility. RT-PCR for ~71% of these 108 genes (e.g. 77 genes) indicated low RNAi efficiency (residual expression > 40%; Figure 1B), suggesting that the currently available TRiP lines did not permit full evaluation of those genes' roles in oogenesis or early embryogenesis. Ovary expression of eight genes was too low to be detectable under our RT-PCR conditions, making it difficult to assess their knockdown efficiency. For the 23 genes in Class 1 that knocked down to 40% or less of residual expression, the lack of phenotype might mean that they are not uniquely essential for female fertility, but we cannot rule out that the residual gene product was sufficient for their function.

Genes whose knockdown affect oogenesis

For the nine genes in Class 2, depletion of their products led to slight reductions in egg production, but did not significantly influence the hatchability of the eggs. These genes may be involved in oogenesis, but their maternally-derived products are not

essential thereafter. It is also possible that their expression was not sufficiently knocked down to fully block oogenesis (or to show later effects).

Genes in Class 3 and Class 4 are likely crucial for oogenesis since knockdown of their expression throughout oogenesis led to severe reduction or complete abolition of egg production. We examined the ovaries of the females knocked down for these genes and found defects at various stages of oogenesis (Figure S2.1, Table S2.3), validating that perturbing the expression of these genes in female germline indeed disrupts various aspects of oogenesis. A summary of the ovary phenotypes can be found in Table S2.3. For the 21 genes in Class 3, restricting depletion of their gene products to mid and late oogenesis by driving knockdown with the matα4-GAL-VP16 driver circumvented the impacts of their knockdowns on egg production and revealed various hatchability phenotypes. This subset of genes is thus likely important in early oogenesis, as well as in egg activation or embryogenesis, but their expression may not be essential in mid and late oogenesis. However, we cannot rule out the possibility that perdurance of protein products from early stages of oogenesis masked any defects from the knockdown in later stages of oogenesis, or that knockdown of these genes driven by *matα4-GAL-VP16* was insufficient to generate an oogenesis phenotype.

For the 22 genes in Class 4, temporal manipulation of their knockdown did not alter the impact of these knockdowns on egg production. Thus, expression of these genes appears to be necessary throughout oogenesis for normal egg production, and it is impossible to determine whether they also affect egg activation or early embryogenesis. Knockdown of *Acn* significantly impacted both egg production and hatchability when driven by either driver, indicating its involvement in late oogenesis

as well as egg activation or embryogenesis. Hence, we assigned *Acn* to a different class, Class 5.

Genes whose knockdown affect hatchability but not oogenesis

For the 28 genes in Class 6, knocking down their expression throughout oogenesis significantly reduced or abolished the hatchability of the fertilized eggs without impacting egg production, suggesting that these genes' activity is essential in egg activation and/or embryogenesis but not for oogenesis (Table 2.1, Figure 2.1A, Table S2.1).

To rule out effects of potential off-targets on our results, we obtained all available shRNA lines targeting a given gene. Of the 16 sets of shRNA lines that targeted the same genes, 11 generated the same phenotype in all lines in a set (Table S2.1). For the other 5 sets of shRNA lines, fertility phenotypes were observed in some lines but not the others in a given set. For four of those five sets, the difference in phenotypes was most likely due to differences in knockdown efficiencies, since the lines with no fertility defects showed the highest levels of residual expression. The two shRNA lines targeting *Nap1* generated similar levels of knockdown, thus the difference in phenotypes may be a result of small differences in knockdown that were undetectable by our semi-quantitative methods, to off-target effects of one of the constructs, or to unknown genetic or environmental factors that varied between the lines and their tests. Taken together, the combination of MTD-Gal4 and *matα4-GAL-VP16* driven germline-specific RNAi identified 53 genes with apparent roles in oogenesis. This group includes factors known to mediate essential processes in oogenesis, such as *14-3-3ε (Benton et al 2002)*, *no child left behind* (*nclb*) (Casper et al 2011), *CG9556* (Pan

et al 2014) and *short stop* (*shot*) (Roper & Brown 2004), which validated the effectiveness of our method at capturing genes needed for egg production. Intriguingly, 32 of the 53 genes we found had no previously reported roles in oogenesis. These genes likely represent new factors in oogenesis that merit further investigation.

We also found 51 genes that are likely to be involved in egg activation and/or embryogenesis. The localization and/or cellular functions of the product of several of these 51 genes, including Su(var)205 (Zhao & Eissenberg 1999, Zhao et al 2001), Modulo (Perrin et al 1999), Otefin (Ashery-Padan et al 1997, Habermann et al 2012) and Axin (Tacchelly-Benites et al 2018, Willert et al 1999, Yamamoto et al 1999), are known to be regulated through phosphorylation. Notably, 22 of these genes have no previously characterized maternal phenotypes.

Maternal knockdowns of 27 genes caused developmental arrest in early embryogenesis.

To investigate the roles of the maternal proteins whose knockdowns caused hatchability phenotypes (Classes 3, 5, and 6), we examined the phenotypes of 2-4-hr old embryos produced by RNAi females fertilized by wildtype (ORP2) males. Genes whose knockdown reduced the hatchability of the embryos to 50% or below were prioritized (43 genes).

D. melanogaster embryos go through 14 syncytial mitotic divisions (embryogenesis stage 1-4) in the first 2 hrs of development, before cellularization takes place at stage 5 of embryogenesis. Embryonic development before cellularization is largely maternally driven (Campos-Ortega & Hartenstein 1997). At 2-4 hrs post fertilization, more than

80% of fertilized embryos produced by control females developed to stage 4 or later stages of embryogenesis (Table S2.4), whereas in the knockdown embryos, we observed developmental arrest at various earlier stages of embryogenesis (Figure 2.2, Figure S2.2, Table S2.4). We were especially interested in knockdowns that led to arrest at stage 1 (apposition of male and female pronuclei) or 2 (syncytial mitotic division 1-8) of embryogenesis, since early arrest likely reflects defects in egg activation or in the transition into zygotic syncytial divisions.

We observed that for 27 genes, maternal knockdown caused a significant portion of the embryos to arrest in stage 1 and/or early stage 2 of embryogenesis. Knockdown of 17 of these genes caused arrest in stage 1 of embryogenesis, prior to completion of first mitosis: *DNA ligase I* (*Dlig-I*) (83% arrested, n = 36)*, minichromosome maintenance* (*mcm3*) (80% arrested, n = 25)*, spindly* (54% arrested, n = 26), *spc105r* (91% arrested, n = 34), *ballchen* (*ball*) (89% arrested, n = 28), *BRWD3* (38% arrested, n = 60), *mbs* (40% arrested, n = 30), *mri* (87% arrested, n = 31), *pyruvate kinase* (*pyk*) (46% arrested, n = 48), *bre1* (43% arrested, n = 35), *mod(mdg4)* (58% arrested, n = 26), *slender lobes* (*sle*) (30% arrested, n = 27), *aubergine* (*aub*) (68% arrested, n = 28), *αTub67C* (96% arrested, n = 25), *acn* (36% embryos, n = 28), *spaghetti (spag)* (13% arrested, n = 72) *and modulo* (*mod*) (24% arrested, n = 55) (Table S2.4), indicating roles of these genes in egg activation or in initiation of syncytial division. Maternal knockdown of 17 of the 27 genes caused significant embryogenesis arrest in early stage 2: *TER94* (86% arrested, n = 21), *spindly* (38% arrested, n = 26)*, 14-3-3ε* (86% arrested, n = 21), *pzg* (91% arrested, n = 22), *mbs* (40% arrested, n = 30), *su(var)205* (40% arrested, n = 15), *PyK* (52% arrested, n = 48)*, mod(mdg4)* (42%

arrested, n = 26), *aub* (32% arrested, n = 28), *sle* (67% arrested, n = 27), *MCPH1* (87% arrested, n = 23), *ana1* (100% arrested, n = 20), *pk92B* (42% arrested, n = 33), *plutonium* (*plu*) (71% arrested, n = 21), *spag* (19% arrested, n = 72), *CG8223* (61% arrested, n = 33), *CG3689* (40% arrested, n = 48). Note that the knockdown of seven genes: *mbs*, *sle*, *mod(mdg4)*, *spag*, *spindly*, *PyK* and *aub*, led to significant arrest in both stage 1 and stage 2 of embryogenesis; thus, they are included in both categories here. In addition, the knockdown of *eco* caused significant developmental arrest in late stage 2 (63% embryos) of embryogenesis.

We confirmed that the lack of hatchability was not due to failure of fertilization, by testing for the presence of a sperm tail in 0.5-1.5-hr old embryos produced by knockdown females (Karr 1991). We found no outstanding reduction in fertilization rate in any of the knockdown embryos (Figure S2.3, Table S2.5). Examination of the sequences of these 27 maternal effect genes revealed that many encode proteins with consensus phosphorylation sites for conserved kinases that are involved in or modulated during *Drosophila* egg activation, including Cdk1 (deactivated upon egg activation (Swan & Schupbach 2007)), Erk (activity decreases upon egg activation (Sackton et al 2007)) and GSK3 (activity required for egg activation in Drosophila (Takeo et al 2012)) (Table S2.6). The presence of these consensus sites suggests that these proteins may be targets of these kinases, consistent with their phospho-modulation during this transition, and suggesting the functional importance of their phosphoregulation at this time. Several of the proteins also contain consensus sites for CaMKIIγ, the calcium dependent kinase that plays essential roles in mammalian egg

activation (Backs et al 2010), but has not yet been tested for involvement in Drosophila egg activation.

Twelve of the 27 genes have previously reported maternal effect phenotypes, validating the premise of our screen that the phosphoregulated proteins include ones whose action is essential during this transition. These 12 genes include *ball* (Ivanovska et al 2005, Nikalayevich & Ohkura 2015), *plu* (Elfring et al 1997, Shamanski & Orr-Weaver 1991), *mod(mdg4)* (Buchner et al 2000), *spc105r* (Radford et al 2015, Yan et al 2014), *mri* (Krauchunas et al 2012), *su(var)205* (Kellum & Alberts 1995), *pk92B* (Sopko et al 2014), *αtub67C* (Komma & Endow 1997, Matthews et al 1993), *MCPH1* (Brunk et al 2007), *mbs* (Sopko et al 2014), *aub* (Mani et al 2014), and *14-3-3ε* (Perrimon et al 1996). In most cases, the knockdown phenotypes we observed recapitulated the previously reported maternal effect phenotypes; the few small discrepancies are likely due to incomplete RNAi efficiency. As an example, *ball* encodes a kinase that is crucial for the condensation of chromosomes during meiosis (Nikalayevich & Ohkura 2015). We observed that Ball depletion in the female germline caused 89% of her embryos to arrest before completing their first mitosis, with aberrant chromosome arrangements in the polar body. This resembles the previously reported phenotype observed in embryos produced by *ball* mutant females (Ivanovska et al 2005, Nikalayevich & Ohkura 2015). Our recapitulation of phenotypes reported in previous studies using germline clones, RNAi, or hypomorphs validated the effectiveness of our methods in detecting genes with maternal effect phenotypes. A full summary of the phenotypes observed in maternally depleted embryos for the 27 genes can be found in Table S2.4.

Intriguingly, for 15 of the 27 genes no maternal effect phenotype had previously been reported in Drosophila. We discuss these genes in more detail below. Thirteen of these genes had previously been shown to affect cellular processes later in development; our results show that they are also critical in the germline for the initiation of embryogenesis. The phosphorylation state change of the maternal product of these genes upon egg activation reflects that they may be actively manipulated to adapt changes of roles during the transition from oocyte to embryo. This group also include two novel factors, CG3689 and CG8223, whose functions are not yet known in Drosophila. Our results revealed their essential involvement in early embryogenesis as maternal effect factors.

Depletion of newly identified maternal effect factors cause spindle defects in early embryos.

To further evaluate the functions of the maternal products of the 15 genes in early embryos, we examined the defects in 2-4 hr old embryos produced by females with knockdown of each of these 15 genes (Figure 2.2, Table S2.4).

Strikingly, for all 15 genes, depletion of their products from the female germline resulted in metaphase spindle structure abnormalities in early embryos, including multi-polar and anastral spindles (Table S2.7, Figure 2.3A). Since both types of defect are associated with abnormal centrosome activity, we examined the presence of centrosomes in the knockdown embryos by staining for the centrosome component γtubulin (Moritz et al 1995).

Intriguingly, multi-polar spindles with supernumerary centrosomes were observed in 73% of *TER94* and 38% of *CG3689* maternal knockdown embryos (Figure 2.3B),

suggesting that over-replication of centrosomes may be associated with aberrant spindle formation in these embryos. Consistent with this observation, we also found excessive accumulation of centrosomes unassociated with spindles in the cytoplasm of these embryos, suggesting uncoupling of centrosome replication from the cell cycle. TER94 is a VCP family protein that is involved in microtubule organization and mRNA localization during Drosophila oogenesis (Ruden et al 2000). However, its roles in early embryogenesis are unclear. CG3689 is the fly ortholog of mammalian NUDT21, which is an RNA processing factor that regulates differential mRNA polyadenylation in differentiating stem cells (Brumbaugh et al 2018, Dettwiler et al 2004, Ruegsegger et al 1998, Yang et al 2010). The function of CG3689 in Drosophila is yet unknown beyond our finding that it is required maternally for mitotic spindle organization in the early embryo.

γtubulin-positive centrosomes also accumulated in the embryos produced by *Sle* RNAi females. However, instead of spindles with supernumerary centrosomes, we found a mixture of normal spindles and spindles with no or one single centrosome in all *Sle* knockdown embryos (Figure 2.2, Figure 2.3A), suggesting that even though centrosomes over-replicated in these embryos, they were not able to properly interact with chromosomes to form mitotic spindles. Sle is known to be involved in nucleolar organization and rRNA processing in neuron and nurse cells (Orihara-Ono et al 2005). Our results reveal a novel function for Sle, as a maternal protein that is crucial for the mitotic divisions in early embryogenesis.

Spindles with single centrosomes were also observed in embryos from females knocked down for *Mcm3* (46%), *Acn* (40%), *BRWD3* (30%), *pzg* (29%), *Dlig-I* (28%),

Bre1 (27%), *spindly* (22%), *CG8223* (14%), *mod* (12%) and *CG3689* (11%) (Figure 2.3B), suggesting abnormal centrosome-chromosome interaction in these knockdown embryos.

Another commonly observed spindle defect in the early embryos from maternal knockdown of these 15 novel maternal effect genes is an acentrosomal spindle. We found bipolar spindles with no centrosomes at spindle poles in large portions of embryos with maternal knockdown of *Ana1* (100%), *PyK* (94%), *Spag* (74%), *Spindly* (70%), *Bre1* (63%), *Mod* (53%), *pzg* (52%), *Mcm3* (46%), *Acn* (45%), *Dlig-I* (44%), and *BRWD3* (30%) (Figure 2.3B). The absence of centrosomes at spindle poles may be a result of unsuccessful centrosome replication or aberrant interaction between centrosomes and chromosomes. Among this group of genes, Ana1 is a centrosome component known to be present in the sperm's giant centriole and proximal centriole-like structure (Blachon et al 2009). Our result suggests that maternal contribution of Ana1 is crucial for the replication of centrosomes in early embryos. Other genes in this group are not known to be involved in centrosome activities or spindle formation.

Maternal depletion of *PyK* affected meiosis completion

Since acentrosomal spindles are characteristic of meiotic spindles, another possibility for the presence of abnormal spindles in these embryos is that meiosis is not completed. We thus examined the status of meiosis completion in the knockdown embryos.

In *Drosophila melanogaster*, completion of meiosis in activated eggs is accompanied by the fusion of three polar bodies into a rosette structure, with condensed chromosomes surrounded by microtubules (Mahowald et al 1983). Therefore, we

searched for the presence of a polar body rosette in 0.5-1.5hr old embryos as a signature of meiosis completion (Table S2.5).

Normal polar body rosettes were observed in 93% of the embryos produced by control (AttP2) females (Figure 2.4). For 9 of the 15 genes, we found no major aberrations in the presence or morphology of polar body rosettes in the embryos produced by RNAi females (Table S2.5). But strikingly, polar body rosettes were absent in large proportions of embryos from females with germline knockdowns of *PyK* (65% absent, n = 31) consistent with the scenario of incomplete meiosis. *PyK* encodes pyruvate kinase, an important component of the glycolytic glycolysis pathway. Our results suggested that glycolysis, and its regulation, may be essential for egg activation.

We also found prominent abnormalities in polar body morphology in embryos with maternal knockdown of *Dlig-I* (83% abnormal, n = 23), *mcm3* (56% abnormal, n = 36), and *spindly* (53% abnormal, n = 32) (Figure 2.4), indicating possible defects in chromosome condensation and microtubule organization during the formation of polar body rosettes in these knockdown embryos.

A complete summary of polar body rosette presence can be found in Supplementary Table S2.5.

Maternal knockdown of *Acn* leads to defects in the crosslinking of egg coverings.

Early embryogenesis arrest may reflect defects in egg activation or in the transition into zygotic syncytial divisions (or both). Thus, we investigated whether the maternal knockdowns that caused early developmental arrest had any impacts on egg activation events, in addition to meiosis completion. Specifically, we examined two other major

events of egg activation in the embryos produced by the knockdown females: changes in egg coverings and translation initiation.

In *Drosophila melanogaster*, egg activation is accompanied by the cross-linking of the vitelline membrane outer layer and the chorion, causing the egg coverings to become impermeable to small molecules (Heifetz et al 2001), and renders the eggs resistant to rapid lysis (in <2 min) in 50% bleach. Thus, to examine whether hardening of the egg coverings was affected by any of the 27 early arrest genes, we examined the lysis of 0.5-1.5-hour-old embryos from knockdown (and control) females after incubation in 50% bleach. We found that the maternal knockdown of *Acn* caused a significant reduction in bleach resistance; only 41% of eggs produced by *Acn* knockdown females persisted longer than 2 min in 50% bleach (Table S2.5), suggesting a role of Acn in facilitating eggshell production or mediating its cross-linking. Maternal knockdown of the other 26 genes did not significantly affect the bleach resistance of the eggs. *Acn* is important for endosomal trafficking and for the stabilization of early endosomes (Haberman et al 2010). It is also involved in RNA splicing as an accessory factor of the exon junction complex (Hayashi et al 2014).

Egg activation is also accompanied by a dramatic increase in the translation of many maternal mRNAs important for subsequent embryogenesis (Horner 2007, Kronja et al 2014b), including (in Drosophila) *smaug* (*smg*), *bicoid* (*bcd*) and *zelda* (*zld*) (Driever & Nusslein-Volhard 1988, Eichhorn et al 2016, Tadros et al 2007). We thus examined the translation of *smg* in activated eggs as a marker of a general increase in translational activities. Since Drosophila egg activation is independent of fertilization (Horner & Wolfner 2008a), we used 0-1hr activated but unfertilized eggs produced by

knockdown females for this experiment to avoid the confounding influences of fertilization and subsequent zygotic development. As expected, Smg protein was detected in the lysate of activated eggs but not in lysates of stage 14 oocytes produced by control females (Tadros et al 2007). We did not detect Smg protein in activated eggs produced by females knocked down for *plu*. This was expected, because *plu* encodes a subunit of the Pan Gu Kinase that is essential for translation of Smg upon egg activation (Tadros et al 2007). In contrast, we found no significant disturbance of *smg* translation in eggs knocked down for any of the other 26 maternal knockdowns, indicating that none of those genes are essential for the translational increase that normally occurs upon egg activation (or for the translation of Smg specifically) (Figure S2.4). We cannot rule out the possibility that the 26 genes whose knockdown did not affect *smg* translation may include factors that regulate the translation of specific maternal mRNAs other than *smg*, or that their knockdown level was insufficient to give a detectable effect on *smg* translation.

2.5 Discussion

In recent decades, germline-specific RNAi screening of genes with enriched germline expression has been used in several studies to identify genes that are important for female fertility. An RNAi screen of this type in *C. elegans* revealed that 322 out of 766 genes with ovarian enriched expression are required for normal egg production and/or embryogenesis (Piano et al 2002). A similar screen in Drosophila found that 10.5% of the 3491 genes with germline-enriched expression were important for female fertility (Yan et al 2014). However, since not all genes that are important for female fertility are preferentially expressed in the female germline, limiting screens to

germline-enriched genes may miss important regulators of female fertility that are more uniformly expressed, such as genes that are required for cell-essential function in the soma later in development. Our study addressed this limitation by searching for maternal functions among proteins that are phosphoregulated during egg activation. Of 189 candidate genes, we identified 81 that are crucial for different aspects of female fertility, indicating that this set of phosphoregulated maternal proteins is a rich candidate pool of important modulators of oogenesis and early development.

Our screen identified 27 genes that encode essential maternal proteins for the egg-to-embryo transition or early in embryogenesis. This set of genes includes several that are essential for cellular processes including mitosis, DNA damage repair and replication in later somatic cells, but for many this is the first report that they also exert these functions maternally, or in the germline. Since early embryogenesis in Drosophila takes place with little transcription activity, it is possible that the products of these essential genes need to be provided maternally to facilitate cellular functions at these early stages.

In this screen, we did not find new germline-specific regulators of female fertility, but rather we revealed germline functions for potentially ubiquitous proteins. Although genetic screens have identified some female-germline-specifically expressed molecules that are essential for the egg-to-embryo transition, such as *fs(1)YA* (Lin & Wolfner 1991, Liu et al 1995), *gnu* (Freeman & Glover 1987, Renault et al 2003), *plu* (Elfring et al 1997, Shamanski & Orr-Weaver 1991), *png* (Hara et al 2017, Shamanski & Orr-Weaver 1991), and (almost exclusively) *wisp* (Benoit et al 2008, Brent et al

2000, Cui et al 2008, Lim et al 2016), our results indicate that this type of gene is rare and that most of the critical molecules for this transition are expressed, and often essential, at other stages. Detection of those molecules among proteins whose phosphorylation state changes during egg activation suggests that the activity of these essential maternally provided factors may be regulated post-translationally during the transition from oocyte to embryo to allow them only to be active at the appropriate time. This will be a fruitful area for future molecular study.

In addition to discovering new, maternal, roles for known proteins, we found two novel factors, CG3689 and CG8223, to be important for early syncytial divisions. Both proteins are conserved in mammalian systems. The mammalian ortholog or CG3689 is Nudt21, which plays a role in the 3' polyadenylation of pre-mRNAs (Dettwiler et al 2004, Ruegsegger et al 1998, Yang et al 2010). CG3689 shares 77% amino acid sequence homology as Nudt21. Since maternal transcripts are dynamically controlled through poly(A)-tail length in late oogenesis and early embryogenesis (Benoit et al 2008, Brent et al 2000, Cui et al 2008, Lim et al 2016), it is possible that CG3689 plays a similar role to Nudt21 We frequently observed misshapen multi-polar spindles in *CG3689* knockdown embryos. Interestingly, embryos from females lacking *Wispy*, a Drosophila poly-(A) polymerase that is known to mediate polyadenylation of maternal transcripts upon egg activation, also arrest in early embryogenesis with abnormal spindle formation (Brent et al 2000, Cui et al 2008). If CG3689 plays a similar molecular role in Drosophila to Nudt21 in mammals, perhaps maternal transcripts of some spindle-associated proteins are regulated by CG3689, and the phenotype that we observe in its knockdown is due to the perturbation in production of

some of these spindle-associated proteins. Though we did not detect abnormalities in Smg translation in CG3689 maternal knockdown, we cannot rule out that its function affects transcripts other than *smg*. This possibility is supported by the finding that Nudt21 binds to RNA at specific sequences (Yang et al 2010). Examination of the global changes in maternal mRNA poly-A tail length upon egg activation in CG3689 depleted background will be informative to elucidate the functions of CG3689.

The mammalian ortholog of CG8223 is NASP (29% amino acid sequence identity), which is, surprisingly, a sperm-specific protein. NASP is a N1/N2 family histone chaperone involved in nucleosome assembly after DNA replication is completed (Finn et al 2008, Osakabe et al 2010, Richardson et al 2006). This suggests interesting possibilities for the role of *CG8223* in Drosophila, as the phenotype we observed in *CG8223* knockdown embryos is consistent with disruptions in chromosome organization or chromatin assembly. Intriguingly, our finding suggests that this novel protein may be particularly important in early embryogenesis, as its depletion in early oogenesis did not seem to disrupt the formation of 16-cell germline cysts.

We were also intrigued to discover 13 genes that are required in early oogenesis and are also important for egg activation or early embryogenesis. Five of these genes encode factors involved in chromatin organization, including *su(var)205* (Hines et al 2009), *pzg* (Eggert et al 2004, Gan et al 2011), *BRWD3* (Chen et al 2015), *ball* (Aihara et al 2004, Ivanovska et al 2005) and *bre1* (Bray et al 2005, Xuan et al 2013). The activity of these factors may need to be regulated appropriately to allow the dynamic reorganization of chromatin landscape that accompanies the differentiation of the oocyte during oogenesis (Boija & Mannervik 2015, Iovino 2014), followed by

conversion to totipotency during egg activation. Understanding how phosphoregulation modulates the activities of these factors and the kinases and other pathways involved in the phosphoregulation of these factors in response to the calcium signal that initiates egg activation will give valuable information about the mechanisms that facilitate the transition between differentiated and totipotent cellular states.

We also found evidence that the function and regulation of glycolysis are essential for egg activation since PyK appears to be dephosphorylated during egg activation (Krauchunas et al 2012), and its depletion led to defects in meiosis completion. A recent study reports a drastic shift of metabolic state in late oogenesis, which is later reversed in the early embryo, pointing out that remodeling of metabolic state may be an important part of the transition from oocyte to embryo (Sieber et al 2016). Since PyK is a component of the glycolysis pathway, its phosphoregulation may be a part of the mechanism that mediates the metabolic changes that take place during egg activation.

Guo et al. (2015) observed 174 proteins in common between our dataset of proteins phospho-modulated during Drosophila egg activation (Krauchunas et al. 2012) and their set of phosphoregulated proteins during this same transition in sea urchin. This led them to suggest that there are conserved cassettes of protein functions needed for this important developmental transition. With this in mind, we find it intriguing that 88% of the genes that our RNAi screen identified as needed for the transition from egg to early embryo in Drosophila have homologs in mouse. Interestingly, the mouse orthologs of seven of these genes (*ball* (mouse *vrk1*) (Wiebe et al 2010), *MCPH1*

(mouse *mcph1*) (Liang et al 2010), *14-3-3ε* (mouse *ywhae*) (Kosaka et al 2012), *rec* (mouse *mcm8*) (Lutzmann et al 2012), RhoGap68F (mouse Arhgap1) (Wang et al 2005), *spindly* (mouse *spdl1*) (Zhang et al 2010) and *CG9556* (mouse *cops2*) (Lykke-Andersen et al 2003)) have been reported to be important for female fertility, consistent with the idea of conserved functions in female reproduction. For the vast majority of the Drosophila fertility factors uncovered by our screens, we do not yet know the roles of their orthologs in female fertility in other organisms, and they are good candidates for investigation as regulators of egg activation or early embryogenesis in organisms beyond Drosophila.

For 108 of the genes that we tested, depletion of their products in the female germline did not impact female fertility. There are numerous explanations for this result, beyond the possibility that these genes might not be essential in these processes. First, in more than 71% of the cases, the knockdown efficiency of these RNAs was too low to allow definitive conclusions about gene function. Although in the future one could imagine circumventing this problem with screens using targeted editing with CRISPR/Cas9, at present those techniques are not sufficiently efficient to allow homozygous germline-specific knockouts. Second, even in the cases of high knockdown efficiency, lack of phenotype could reflect functional redundancy. Third, it is possible that some of these genes encode proteins that are not essential for oogenesis, but must be deactivated upon egg activation, or proteins that are needed to prevent premature activation of oocytes; neither type would have been detectable in our screen. Finally, the 311 proteins identified by Krauchunas et al 2012 are unlikely

to represent the entire set of proteins that are phosphoregulated upon egg activation since even the newer improved mass spectrometry currently available (5 years after that study) still cannot comprehensively detect all peptides in a sample. Therefore, additional essential factors that are phosphoregulated at egg activation likely remain to be discovered.

2.6 Aknowledgement

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Table 2.1. Classification of fertility phenotypes in the primary and secondary germline specific rnai screen.

Class	MTD-Gal4*		matαtub-Gal-VP16**		Number of genes	Interpretation
	Egg production	Hatchability	Egg production	Hatchability		
Class 1	Normal	Normal	NA	NA	108	RNAi efficiency; not essential
Class 2	Reduced	Normal	NA	NA	9	Oogenesis
Class 3	Severely reduced	NA	Normal	Reduced	21	Early oogenesis; egg activation/ embryogenesis
Class 4	Severely reduced	NA	Severely reduced	NA	22	Mid to late/ throughout oogenesis
Class 5	reduced	reduced	reduced	reduced	1	Mid to late/ Throughout oogenesis; egg activation/ embryogenesis
Class 6	Normal	Reduced	NA	NA	28	Egg activation/ embryogenesis

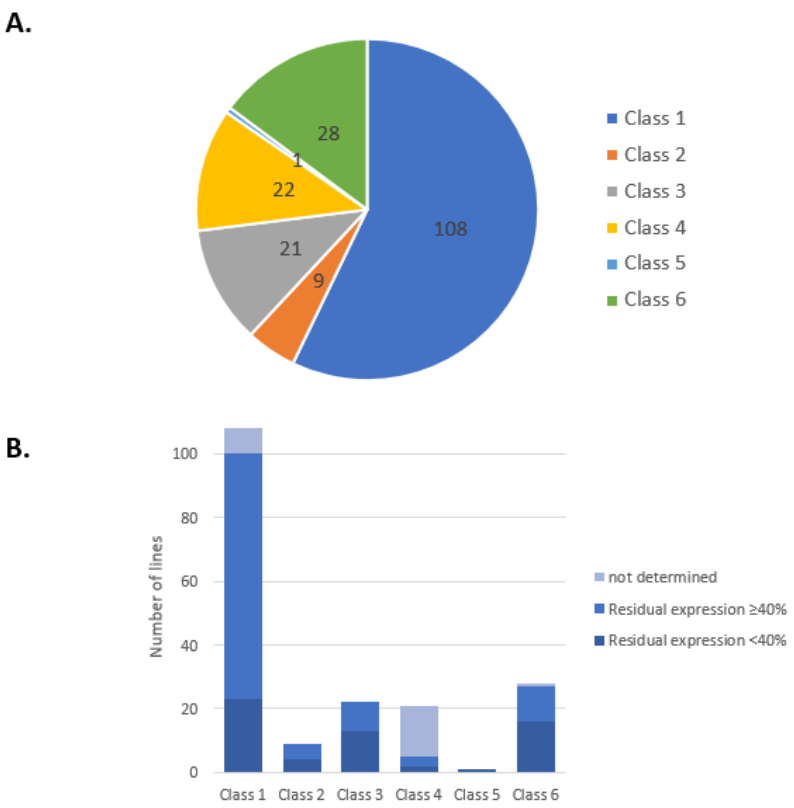

Figure 2.1 Germline-specific RNAi knockdown of maternal proteins that are phosphoregulated upon egg activation exhibited different impacts on female fertility. (A) The frequency of various fertility phenotypes observed in RNAi females (MTD-Gal4/UAS-shRNA or matαrub-Gal4/UAS-shRNA) crossed to ORP2 males. A total of 189 genes were screened. (B) Knockdown efficiency of the target gene in different phenotype classes. The number of genes in each phenotype class with residue expression level equal/below 40%, greater than 40% are plotted. The residue expression of the target genes in the oocytes produced by RNAi females was examined using RT-PCR and band intensity quantification. Band intensity of the target amplicon was normalized to that of Rpl32 in control and RNAi samples. The expression of genes cannot be evaluated when the knockdown led to abolished egg production due to the lack of oocyte. The expression of 9 genes cannot be detected with RT-PCR

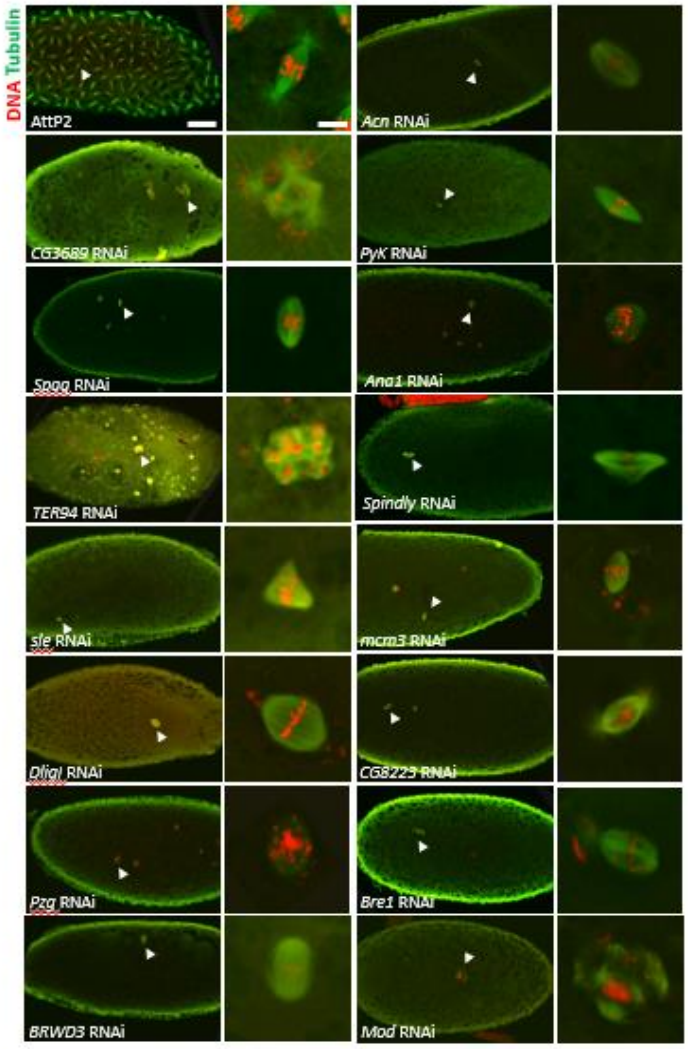

Figure 2.2 Maternal knockdown of 15 genes led to developmental arrest in stage 1 and/or early stage 2 of embryogenesis. 2-4 hr old embryos from knockdown females, stained for tubulin (green) and DNA (red). For each set of images, the one on the right shows a close-up view of the spindle (marked by the arrow on the left). embryos from AttP2 control females mated with ORP2 males are used as positive control. Scale bars: embryo 50 µm, nucleus 5 µm.

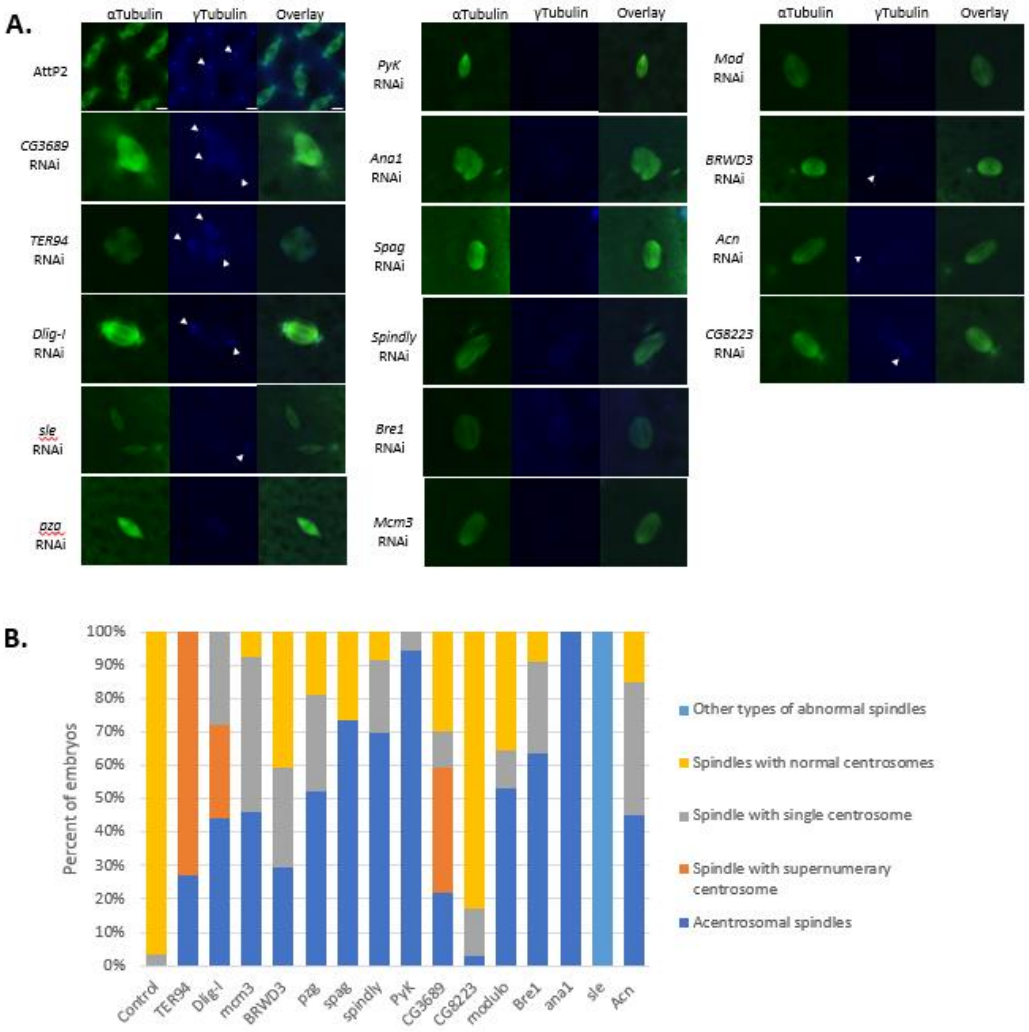

Figure 2.3. Maternal knockdown of 15 factors led to various abnormalities in centrosome activities. (A) Representative images of metaphase spindles in early embryos with normal (AttP2) or abnormal (maternal RNAi knockdowns) centrosome arrangement (white arrows). 2-4hr old embryos are stained for αtubulin (green), and γtubulin (blue). Scale bar: 5μm. (B) Proportion of maternal knockdown or control embryos with normal, supernumerary, acentrosomal spindles or spindle with single centrosome.

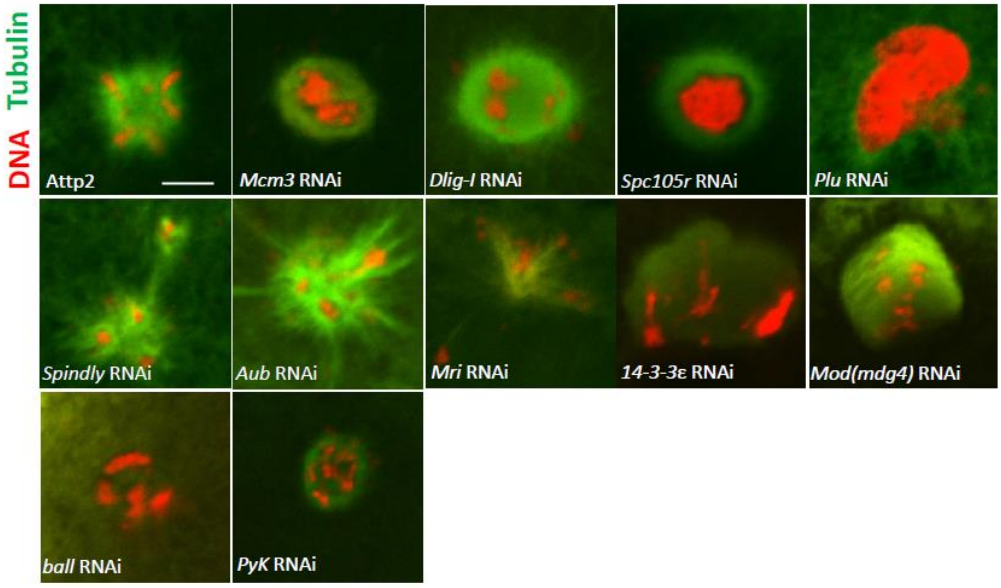

Figure 2.4. Polar bodies with abnormal morphology were observed in knockdown embryos of 11 genes. Significant portions of 0.5-1 hr embryos with maternal knockdown of Dlig-I (83% abnormal, n=23), mcm3 (56% abnormal, n=36), spc105r (96%, n=23), plu (100%, n=30), spindly (53% abnormal, n=32), aub (57%, n=28), mri (62% abnormal, n=37), 14-3-3ε (93% abnormal, n=29), mod(mdg4) (58% abnormal, n=24), ball (88%, n=24) and PyK (13%, n=31) had polar bodies with abnormal morphology. Tubulin is in green. DNA is in red. Scale bars: 5μm

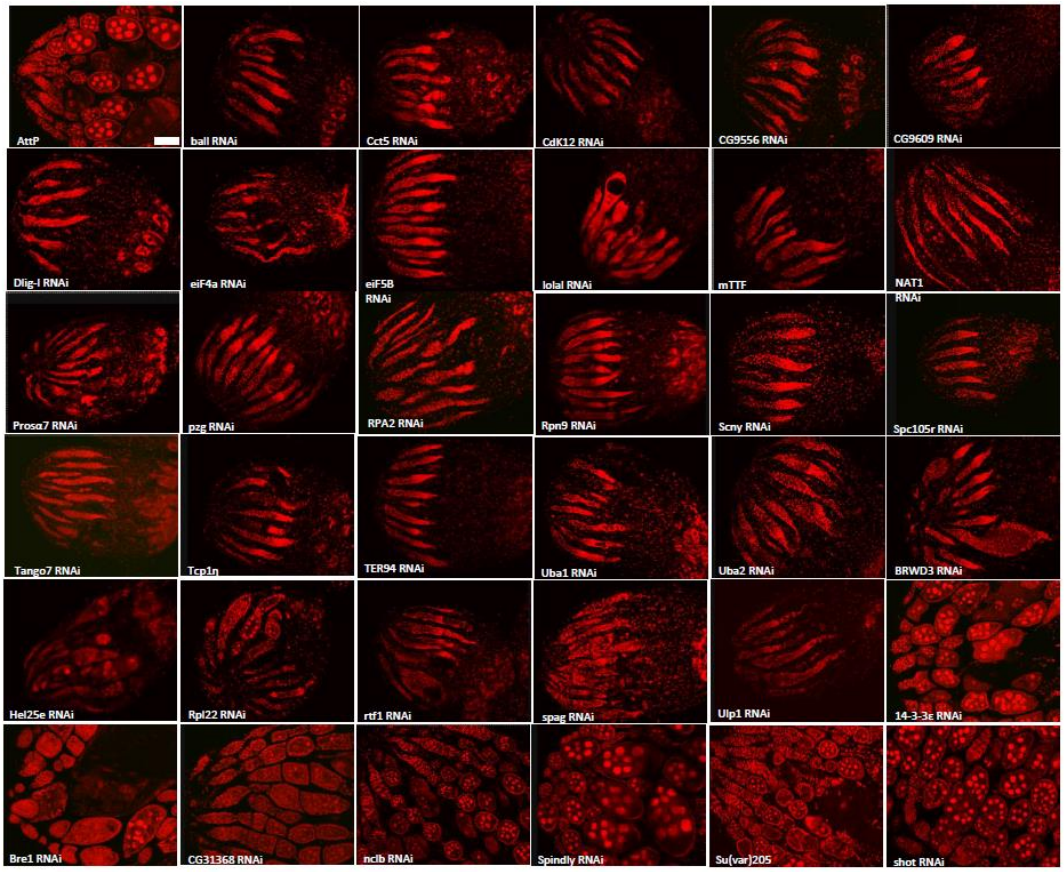

Figure S2.1 RNAi knockdown of Class3 and 4 genes led to defects in various aspects of oocyte development. Ovaries dissected from 3-5 days old control or knockdown females are stained for DNA (red) using propidium iodide. The images are arranged according to observed stage of oogenesis abolishment, from early stage to late stage.

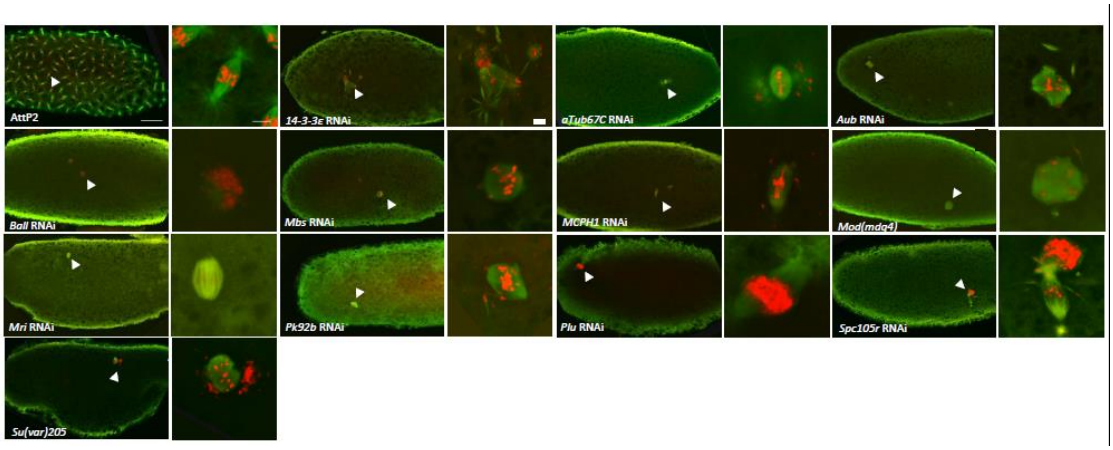

Figure S2.2 Maternal knockdowns that led to early embryogenesis arrest caused various defects in early embryos. 2-4 hr old embryos from knockdown females, stained for tubulin (green) and DNA (red). For each set of images, the image on the right shows a close-up view of the spindle (marked by the arrow on the left). Images are organized in alphabetical order. Scale bar: oocyte image 50 µm, enlarged image 10 µm.

Figure S2.3 Fertilization was not significantly impacted in the early arrest knockdown embryos of the 27 genes Sperm tail (green; arrow) was detected in the stage 1-2 arrested embryos produced by RNAi females for 27 genes. The images are ordered in alphabetical order. Scale bar: 50 μm.

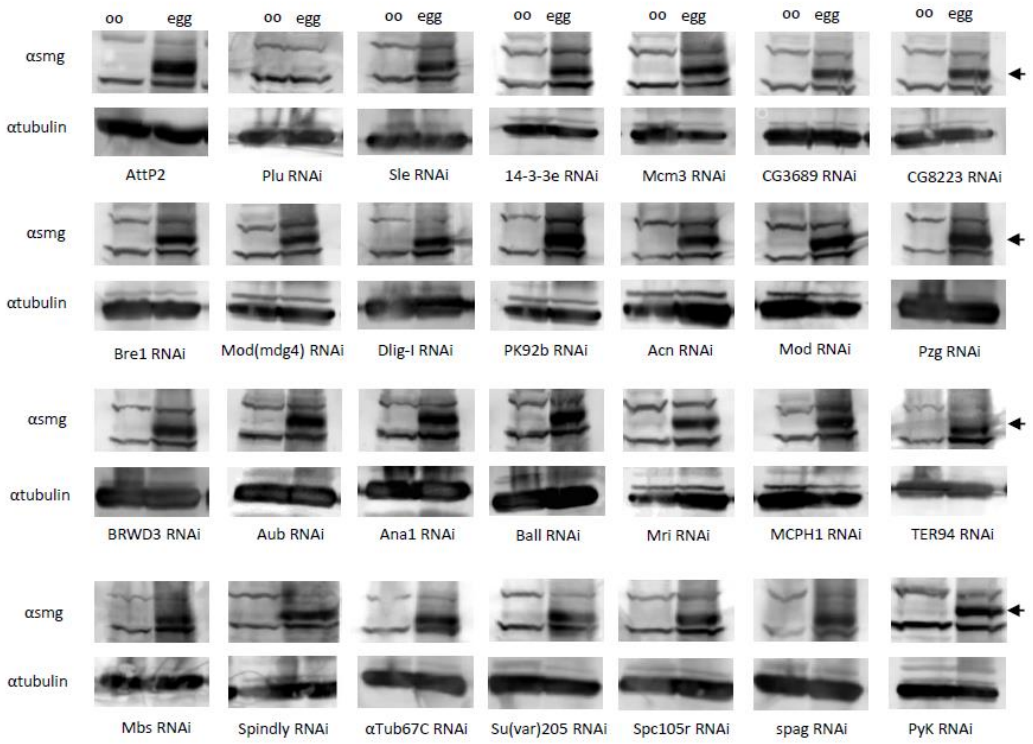

Figure S2.4 Translation of *smg* in unfertilized eggs produced by control and RNAi females of 27 early maternal effect candidates. Among the 27 genes examined, the presence of Smg protein was detected in the activated eggs produced by RNAi females of 26 genes. Translation of Smg protein was not detected in the activated eggs produced by *plu* knockdown females.

Table S2.6 Products of the 27 proteins that are important for early embryogenesis carry consensus phosphorylation sites of conserved kinases that are known to be active or regulated upon egg activation.

Kinase	Protein	Scoring percentile	Sequence motif
Cdc2 (Drosophila Cdk1)	Acn	0.002%	RSRGSRTTPRKRTES
		0.045%	ERAGSGASPVRRSRG
	Ana1	0.025%	PTKSPPRSPRKCVER
		0.047%	APPSPTKSPPRSPRK
		0.086%	CSSTTASSPERRPRK
	Ball	0.167%	VKTEPKSTPRERATP
		0.176%	NGHGDSSTPGRSPRT
	BRWD3	0.042%	GRGGSSSSPNKRVKK
	Dlig-I	0.167%	SDATDSPSPPKKVPK
	Mcm3	0.070%	GEASSQRSPSRRSKR
	MCPH1	0.052%	SELMDISSPRRRSVA
	Mod	0.039%	EETVVPQSPSKKSRK
	Pk92b	0.067%	PTTTTPGTPGIKTKI
	Spc105r	0.145%	DQEEMCKTPIHKRVL
	Sle	0.058%	EKKRFPKTPGREKVP
MAPK1 (Drosophila Erk)	Ana1	0.036%	KRSAKASVELRTTNT
		0.066%	EKQRLVEVTLTSNSD
	Aub	0.070%	KRAVMGMVILTDYNN
	Ball	0.184%	RKKTAKKVTPSARNA
	BRWD3	0.000%	RRRNGVAVSASSSGS
		0.089%	PRLCCLKLAIIDEEG
	Mbs	0.002%	RRISSGPIALNASNQ
		0.079%	ARPTDLPLIPAPAAP
		0.190%	ARDSLLSLYARRTTD
		0.064%	KRRSTGVVHIDMDEL
	Mcm3	0.058%	RRETRRSLPARSVAM
		0.185%	RKNPQRALGLLSNAA
	MCPH1	0.124%	RKLRGMRLDPTVTQH
		0.014%	RRAEDDRVYLDPYWV
		0.190%	RRSSLLGIAKQAEET
	Mod	0.148%	PKVKVGKIPLGTPKN
	Mod(mdg4)	0.158%	CRGDLVDVSLAAEGQ
	Pk92b	0.032%	PRKVRPALPINTEFG
		0.054%	IRKSDLEVLIRGLRD
		0.120%	KRAVQAAVTIFSPEL
	Spc105r	0.079%	REHVDLSVNLQASVD

	Spindly	0.005%	RKGTPVRIPVIKDST
		0.052%	KRNLDTALAAESYLT
	TER94	0.002%	RRKETVCIVLSDDTC
		0.070%	RRIVSQLLTLMDGMK
		0.110%	KKSSHLIVMAATNRP
GSK3A (Drosophila GSK3)	Ball	0.191%	ASPKPRSTPKASPKP
	Mbs	0.146%	RTQAPLSSRRRSLSS
		0.079%	QPPPPPTTPPPAVIP
	Pzg	0.180%	QKDSPNTTAPVSVSI
	Sle	0.109%	EVKQEKPSPSPSPVP
		0.119%	EKPSPSPSPVPKEGK
		0.149%	RKPSVSPSPGRTLTP
	Spag	0.066%	RKESPSSSAASSPTE
CaMKIIγ (Drosophila CaMKII)	CG8223	0.112%	ATALRQSSVLIEEEI
	Pzg	0.063%	YETCKQQTFQCKTCR
	TER94	0.121%	LQLFRGDTVILKGKR
	Spag	0.071%	LCYLKQESFDQCVED
	Spc105r	0.078%	QQIKRRISFSGKKSV
		0.103%	PSAKTQQSIAMSESM
		0.118%	KSIVSQKTIDKSTEQ

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CHAPTER 3

INVESTIGATING THE ROLE OF CALCINEURIN IN EGG ACTIVATION IN DROSOPHILA MELANOGASTER

3.1 Abstract

In all animals studied to date, the crucial process in which an arrested mature oocyte transits into an actively developing embryo (termed egg activation) initiates with a rise of Ca^{2+} level in the oocyte's cytoplasm. This Ca^{2+} rise sets off a series of downstream events, including the completion of meiosis and dynamic remodeling of oocyte transcriptome and proteome, which prepare the oocyte to undertake embryogenesis. Calcineurin is a highly conserved phosphatase that is activated directly by the Ca^{2+} rise upon egg activation in many species. Though calcineurin is known to be required for the resumption and completion of meiosis in Xenopus, ascidian and Drosophila, its specific activity during egg activation and how it transduces the calcium signal to regulate downstream events are still unclear. In this study, we investigated the regulatory role of calcineurin during egg activation in *Drosophila melanogaster*. Using quantitative mass spectrometry, we quantified the phosphoproteomic and proteomic changes that take place during egg activation, and examined how these changes are affected when calcineurin function is perturbed in female germ cells. We showed that calcineurin is required for the proper regulation of hundreds of phosphosites, as well as the abundance changes of numerous proteins during egg activation.

3.2 Introduction

Egg activation is a series of major cellular changes through which a resting mature oocyte transits into an active cellular state that is capable of supporting embryo development. During this transition, the oocyte vitelline membrane undergoes biochemical modifications, the meiotic cell cycle is released from its species-specific arrest (metaphase II in vertebrates and metaphase I in Drosophila) and completes, and the oocyte transcriptome and proteome are remodeled through post-transcriptional and post-translational regulations (Aviles-Pagan & Orr-Weaver 2018, Horner & Wolfner 2008b, Krauchunas & Wolfner 2013, Laver et al 2015, Machaty et al 2017, Marcello & Singson 2010, Von Stetina & Orr-Weaver 2011). These changes are intricately regulated and transform the oocyte from a highly differentiated gamete to a totipotent zygote.

In vertebrates and many invertebrate species, egg activation is triggered by fertilization (Knott et al 2005, Saunders et al 2002, Steinhardt et al 1977, Yoon et al 2012). But in insects, it is triggered by passage through the female reproductive tract and is independent of fertilization (Horner & Wolfner 2008a, Kaneuchi et al 2015, Sartain & Wolfner 2013, Went & Krause 1974, York-Andersen et al 2015). However, despite the difference in trigger event, egg activation initiates with a rise of Ca2+ in the oocyte cytoplasm, which is thought to set off egg activation by activating Ca2+ or Calmodulin (CaM) dependent factors (Sanders & Swann 2016, Sartain & Wolfner 2013). These factors transduce the Ca2+ signal downstream by modulating the activities of maternally deposited proteins through post-translational modifications.

Among post-translational regulation mechanisms, phosphoregulation is thought to be especially important in egg activation. Abundant protein phosphorylation state changes during this transition have been detected by proteomic studies in vertebrates and invertebrates (Guo et al 2015, Krauchunas et al 2012, Presler et al 2017, Roux et al 2006). In Drosophila, more than 300 proteins subject to phosphoregulation during egg activation (Krauchunas et al 2012), more than 55% of which are also phosphoregulated during sea urchin egg activation (Guo et al 2015). These deeply conserved phosphoproteomic changes suggest their functional importance during this transition. The functional studies in Chapter Two revealed that these phosphoregulated proteins include numerous factors that are required for oogenesis and embryogenesis. These results support that phosphoregulation is an important mechanism that remodel the oocyte proteome to prepare the cell to undertake development. However, the pathways that mediate the phosphoregulation of most of these proteins remain unclear. Thus, understanding the activities of phosphoregulators is crucial for elucidating the egg activation process.

Among the effectors of Ca2+, Ca2+/CaM dependent kinase II (CaMKII) and Serine/threonine phosphatase calcineurin are two highly conserved phospho-regulators that have been shown to be crucially involved in aspects of egg activation in different species. In mouse and Xenopus, CaMKII plays an essential role in reinitiating meiosis. It releases the metaphase II arrest by phosphorylating and promoting the degradation of Erp1/EMI2, an inhibitor of E3 ubiquitin ligase Anaphase Promoting Complex/Cyclosome (APC/C) (Backs et al 2010, Ducibella & Fissore 2008). Once activated, APC/C targets Cyclin B for degradation and thus downregulates MPF

promotes metaphase exit. However, in invertebrates, the role of CaMKII in egg activation is either uncharacterized (in Drosophila) or nonessential (in ascidians) (Levasseur et al 2013).

Calcineurin is known to be a crucial player in meiosis resumption and completion in several vertebrate and invertebrate species, but not yet in mammals. Calcineurin acts as a heterodimer, and consists of a catalytic subunit A and a calcium-sensing regulatory subunit B (Rusnak & Mertz 2000). In Xenopus, calcineurin is transiently activated following the first Ca2+ influx, and promotes MII exit by removing the inhibitory phosphorylations on APC/C component Apc3 and its co-activator Fzy (Mochida & Hunt 2007, Nishiyama et al 2007). In Drosophila, oocytes depleted of calcineurin regulatory subunit CanB2 are able to progress from metaphase I arrest upon egg activation, but eventually arrest again in anaphase I (Takeo et al 2010). On the other hand, constitutive activation of calcineurin causes a range of meiotic defects in mature oocytes (Takeo et al 2010), indicating that the function and proper regulation of calcineurin are required for proper meiosis regulation in Drosophila. However, the details of calcineurin activity during egg activation are still unclear.

In addition to meiotic cell cycle regulation, involvement of calcineurin is also implied in other aspects of egg activation in Drosophila. Depletion of the calcineurin regulatory subunit CanB2 inhibits the dephosphorylation and activation of Gnu, and YA (Krauchunas et al 2013). Gnu is a regulatory subunit of Pan Gu Kinase (Freeman and Glover 1987, Renault et al 2003), a crucial regulator of maternal mRNA translation and cell cycle progression in early embryo (Kronja et al 2016). And YA is a maternal effect factor that is essential for the initiation of embryonic mitosis (Lin

and Wolfner 1991, Liu et al 1995, Lopez et al 1994). In addition, the calcineurin regulator Sarah (Sra; calcipressin) is also required at the transition from oocyte to embryo (Horner et al 2006, Takeo et al 2010, Takeo et al 2006). Depletion of Sra leads to anaphase I arrest in activated eggs, and prohibits other events including polyadenylation of crucial maternal transcripts and maturation of male pronucleus (Horner et al 2006, Takeo et al 2006). These findings suggest that calcineurin is at the center of the regulatory mechanisms that drive the transition from oocyte to embryo in Drosophila.

In this study, we investigated the regulatory role of calcineurin during Drosophila egg activation using quantitative mass spectrometry. We analyzed the proteomic and phosphoproteomic changes that take place during egg activation in *Drosophila melanogaster* using quantitative mass spectrometry, and examined how these changes are affected when calcineurin function is perturbed in female germ cells. Our data revealed that calcineurin activity is crucial for the regulation of hundreds of phosphosites, as well as the abundance changes of numerous proteins during egg activation. The set of proteins whose phosphorylation states or abundance are influenced by calcineurin includes important regulators of egg activation events, such as meiotic cell cycle progression and protein translation. We also found enrichment of functional groups related to biological processes in early embryogenesis among these proteins, suggesting that calcineurin may mediate phosphorylation changes that are crucial for early embryo development. Our results revealed the scale of calcineurin's impact on the remodeling of oocyte proteome during egg activation, and provided

molecular evidence that facilitate the understanding of the cellular state in CanB2 kd activated eggs.

3.3 Materials and Methods

Germline-specific RNAi: AttP2 (BL36303), UAS-CanB2 shRNA (BL38971) and nos-GAL4 (BL32563) fly stocks are purchased from Bloomington Stock Center and maintained on standard yeast-glucose-agar media, at 23±2°C, under a 12-hr light cycle.

To generate flies with germline-specific CanB2 knockdown, 5-10 virgin females from UAS-CanB2 shRNA stock were mated to 5-10 males from nos-GAL4 stocks. Virgin daughters generated from the cross were collected (referred to as "RNAi females") and used for sample collection. Virgin daughters generated by AttP2 females mated to nos-GAL4 males were used as control (referred to as "*wt* females"). RNAi and control females were raised and maintained under 27°C. The efficiency of knockdown was validated with RT-PCR (Figure S3.1A, B). Embryos from CanB2 knockdown females were confirmed to be arrested in anaphase I (as explained; Takeo et al 2010) using immunofluorescence (Figure S3.1C).

RT-PCR: Ovaries were dissected from virgin females that were aged for 3-5 days. RNA extraction and RT-PCR were performed as described in Chapter Two. The *CanB2* cDNA was amplified using primers CanB2_F: 5'-AGCGCGTGATCGACATTT, CanB2_R: 5'-CTTGCCATCCTCATCCTTATCC. The control RpL32 cDNA was amplified using primers RpL32_F: 5'-

CACCAGTCGGATCGATATGC, RpL32_R: 5'-CGATCCGTAACCGATGTTG (Findlay et al 2014).

Immunofluorescence: 0.5-1.5 hr old fertilized embryos were collected, fixed and stained as described in Chapter Two. Mouse anti-tubulin (Sigma, St. Louis, MO, catalog #T5168) was used at a dilution of 1:400. Alexa Fluor® 488 conjugated anti-mouse (Thermo Fisher) was used at 1:200. Propidium Iodide was used at 10 µg/ml. Samples were examined and images were generated using a Leica TCS SP2 confocal microscope at the Cornell Imaging Core.

Sample preparation: Mature stage 14 oocytes were dissected from virgin females (aged for 3-5 days) in hypertonic Isolation Buffer (IB) (Page & Orr-Weaver 1997). Activated but unfertilized eggs were collected from females (aged for 3-5 days) that had mated to the spermless sons of *tudor* males. Collection was conducted in 30 min periods using grape juice agar plates.

For each sample, 500-600 oocytes or activated eggs were homogenized in Protease/ Phosphatase Inhibition Buffer (PPIHB) as described in Krauchunas et al (2012). The lysates were centrifuged at 20000xg under 4°C for 30 min, and supernatants were collected. A total of 5 oocyte samples (2 replicates for control and 3 replicates for CanB2 kd) and 5 activated egg samples (2 replicates for control and 3 replicates for CanB2 kd) were prepared. 5 pmol of CSN1S1/CSN2 phosphoprotein standard (Sigma Aldrich) was spiked to each activated egg lysates. The 10 samples were validated by Gnu phosphorylation states using western blot (Figure S3.2) (Hara et al 2017, Krauchunas et al 2013, Lee et al 2003). As reported in previous studies, Gnu slower

mobility form, corresponding to phosphorylated Gnu, was detected in all the mature oocyte lysate and the CanB2 kd activated egg lysates, while the Gnu faster mobility form, corresponding to dephosphorylated Gnu was detected in the control, but not CanB2, activated egg lysates (Hara et al 2017, Krauchunas et al 2013, Lee et al 2003). Protein concentration in each sample was determined by Bradford assay, and further quantified by electrophoresis on a precast NOVEX 10% Bis/Tris mini-gel (Invitrogen, Carlsbad, CA) along with a series of amounts of E. coli lysates (2.5, 5, 10, 15 µg/lane). The SDS gel was stained with colloidal Coomassie blue (Invitrogen), imaged by Typhoon 9400 scanner followed by ImageQuant TL 8.1 (GE Healthcare).
Immunoblotting: 15 µl of each of the 10 samples was mixed with 15 µl of SDS loading buffer. Proteins were separated by electrophoresis in 12% polyacrylamide SDS gels. Primary guinea pig anti-Gnu antibody was used at 1:10000 (Lee et al 2003) (kindly provided by T. Orr-Weaver at Massachusetts Institute of Technology). Secondary HRP conjugated anti-guinea pig was used at 1:1000 (Jackson Laboratories).

TMT labeling: TMT labeling of the proteins was performed according to Thermo Scientific's TMT Mass Tagging Kits and Reagents protocol (http://www.piercenet.com/instructions/2162073.pdf) with a slight modification as described in previous publications by Yang et al and Yang et al (Yang et al 2018, Yang et al 2011). A total of 110 µg protein of each sample.
High pH reverse phase (hpRP) fractionation: The samples were separated into 10 fractions using high pH reverse phase fractionation as described by Yang et al (Yang

et al 2011). Each of the 10 fractions was dried and reconstituted in 100 µL of 2% ACN/0.5% FA for nanoLC-MS/MS analysis.

Phosphopeptide enrichment: TiO2 enrichment was performed using a TiO2 Mag Sepharose kit (from GE Healthcare) according to standard procedures.

Nano-scale reverse phase chromatography and tandem MS (nanoLC-MS/MS): The nanoLC-MS/MS analysis was carried out using an Orbitrap Fusion (Thermo-Fisher Scientific, San Jose, CA) mass spectrometer equipped with a nanospray Flex Ion Source using high energy collision dissociation (HCD) similar to previous reports (Thomas et al 2017, Yang et al 2018) and coupled with the UltiMate3000 RSLCnano (Dionex, Sunnyvale, CA). Each reconstituted fraction (2-3 µL for global proteomics fractions) and the enriched phosphor-fraction (10 µL) were injected onto a PepMap C-18 RP nano trap column (3 µm, 100 µm × 20 mm, Dionex) at 20 µL/min flow rate for on-line desalting, and separated on a PepMap C-18 RP nano column (2 µm, 75 µm x 25 cm). The labeled peptides were eluted in a 120 min gradient of 5% to 35% acetonitrile (ACN) in 0.1% formic acid at 300 nL/min., followed by an 8-min ramping to 95% ACN-0.1% FA and a 9-min hold at 95% ACN-0.1% FA. The column was re-equilibrated with 2% ACN-0.1% FA for 25 min prior to the next run. The Orbitrap Fusion is operated in positive ion mode with nano spray voltage set at 1.6 kV and source temperature at 275 °C. External calibration for FT, IT and quadrupole mass analyzers was performed. For global proteomics fractions, the instrument was operated in data-dependent acquisition (DDA) mode using FT mass analyzer for one

survey MS scan for selecting precursor ions followed by 3 second "Top Speed" data-dependent HCD-MS/MS scans for precursor peptides with 2-7 charged ions above a threshold ion count of 10,000 with normalized collision energy of 37.5%. MS survey scans at a resolving power of 120,000 (fwhm at m/z 200), for the mass range of m/z 400-1600 with AGC = 3e5 and Max IT = 50 ms, and MS/MS scans at 60,000 resolution with AGC=1e5, Max IT = 120 ms and with Q isolation window (m/z) at 1.6 for the mass range m/z 105-2000. Dynamic exclusion parameters were set at 1 within 45s exclusion duration with ±10 ppm exclusion mass width. All data are acquired under Xcalibur 3.0 operation software and Orbitrap Fusion Tune 2.1 (Thermo-Fisher Scientific). For phosphoproteomics fraction, the instrument was operated in either multiplestage activation (MSA)-based Synchronous Precursor Selection (SPS) MS3 method or neutral loss (NL) triggered MS3 method for phosphopeptides (Jiang et al 2017). In MSA SPS MS3 method, orbitrap analyzer was used for acquiring CID MS2 spectra to increase confidence of phosphopeptide identifications and site location while the multiple MS2 fragment ions isolated by SPS were subjected to HCD fragmentation at MS3 level for reporter ion quantitation. In the NL-triggered MS3 method, a regular MS2 spectrum was first generated from the precursor using CID. If the neutral loss peak is detected, then an HCD MS3 scan is acquired on the most abundant ion to obtain both the sequence information and the quantitation.

Data processing, protein identification and data analysis: All MS and MS/MS raw spectra from each set of TMT10-plex experiments were processed and searched using Sequest HT software within the Proteome Discoverer 2.2 (PD2.2, Thermo). The

UnitProt Drosophila Melanogaster database that contained 21,015s entries were used for database searches. The default search settings used for 6-plex TMT quantitative processing and protein identification in PD2.2 searching software were: two mis-cleavage for full trypsin with fixed carbamidomethyl modification of cysteine, fixed 6-plex TMT modifications on lysine and N-terminal amines and variable modifcations of methionine oxidation, deamidation on asparagines/glutamine residues and acetylation of protein N-terminus. The peptide mass tolerance and fragment mass tolerance values were 10 ppm and 50 mDa, respectively. Identified peptides were filtered for maximum 1% FDR using the Percolator algorithm in PD 2.2 along with additional peptide confidence set to high. The TMT10-plex quantification method within Proteome Discoverer 2.2 software was used to calculate the reporter ratios with mass tolerance ±10 ppm with applying the isotopic correction factors. Only peptide spectra containing all reporter ions were designated as "quantifiable spectra" and used for peptide/protein quantitation. A protein ratio was expressed as a median value of the ratios for all quantifiable spectra of the unique peptides pertaining to that protein. A precursor co-isolation filter of 50% was also applied for minimizing ratio compression caused by co-isolation of precursor ions. For each relative ratio group, normalization on protein median was applied. The comparison between groups was undertaken with Microsoft Excel software.

For quantitative phosphopeptides analysis, an additional phosphorylation on Ser, Thr, Tyr residues were specified as variable modifications. To confidently localize phosphorylation sites, the phosphoRS 3.0 node integrated in PD 2.2 workflow was also used. The algorithm of phosphoRS 3.0 software enables automated and confident

localization of phosphosites by determining individual probability values for each putatively phosphorylated site within the validated peptide sequences (Taus et al 2011). For each relative ratio group of phosphopeptides/sites, no normalization was applied.

3.4 Results and discussion

TMT-based quantitative proteomic analysis captured prevalent protein phosphorylation changes in egg activation.

We performed quantitative analysis of phosphoproteomic changes during egg activation in control and CanB2 kd samples (for simplicity, in this paper, mature stage 14 oocyte will be referred to as "oocyte", and activated unfertilized eggs will be referred to as "egg"). Control oocyte and egg lysates (2 replicates each) and CanB2 kd oocyte and egg lysates (3 replicates each) were analyzed together as a TMT-10plex (Figure 3.1). Since the phosphoproteomic analysis is highly susceptible to ratio compression caused by co-isolation interference, we employed two recently developed acquisition methods: multistage activation (MSA) and neutral loss trigger (NL) (Jiang et al 2017) in our MS analysis to improve the accuracy of peptide quantitation.

The MSA method quantified a total of 1472 unique phosphopeptides derived from 714 proteins, while the NL method quantified 1167 unique phosphopeptides derived from 599 proteins (Table S3.1). The two methods together provide quantitative information on the abundance changes of 1856 phosphopeptides from 824 proteins during egg activation.

To evaluate the quality of the datasets, we first assessed the variation of phosphopeptide and protein abundance among the biological replicates by plotting the abundance of phosphopeptides and proteins in each sample against that in each of its biological replicates within the 10plex. We found that phosphopeptide/protein abundance was highly consistent among biological replicates ($R > 0.9$) (Figure S3.2), indicating that our results are highly reproducible.

To compare the phosphoproteome of mature oocyte and activated eggs, we calculated the Log2 egg-to-oocyte peptide abundance ratios for control (4 ratios from 2 replicates) and CanB2 kd (9 ratios from 3 replicates) sample sets. According to the deviations between ratios of biological replicates, we calculated the internal errors of the MSA (0.74 fold) and NL (0.99 fold) datasets, which describes 95% of the variations caused by biological differences and technical among replicates (Gan et al 2007). Therefore, peptide abundance changes greater than the internal errors likely reflect genuine changes in abundances. We thus used the internal errors as the threshold for significant differential regulation. Using one-sample t-tests, we determined that phosphopeptides with abundance changes significantly greater than 0.74 fold ($p<0.05$) in MSA dataset or 0.99 fold ($p<0.05$) in NL dataset are considered to be differentially regulated.

Basing on these standards, the two methods together detected significant abundance changes in hundreds of phosphopeptides (Table 3.1), reflecting dynamic changes in protein phosphorylation states during egg activation. Consistent with previous observations by Krauchunas et al (2012), we detected more peptides whose phosphorylation were downregulated during egg activation than ones whose

phosphorylation were upregulated, suggesting that protein dephosphorylation is more prevalent than protein phosphorylation during egg activation. The number of phosphopeptides with significant abundance changes was notably lower in the CanB2 kd samples in comparison with the control, suggesting that the absence of Calcineurin activity caused aberrant regulation of numerous phosphosites.

Since protein synthesis and degradation also occur during egg activation, to ensure that the changes of phosphopeptide abundances reflect authentic phosphorylation changes, rather than changes in protein levels, we performed global proteomic analysis of the sample sets, and quantified the changes in protein levels during egg activation. We identified a total of 4039 proteins containing at least 2 unique peptides per protein, among which 133 significantly upregulated and 123 significantly downregulated proteins in control activated eggs in comparison to mature oocyte (threshold for significant changes = 0.32 fold, determined by internal error).

In comparisons of the phosphoproteomic and global proteomic data, the vast majority (>80%) of proteins with differentially regulated phosphopeptides remained constant in level during egg activation (Figure 3.2A). Thus, changes in phosphopeptide abundance reflect phosphoregulation, rather than changes in protein abundance, in most cases.

Finally, to validate the efficiency of our method on capturing changes in phosphopeptide abundance, we added 5 pmol of bovine α/β-Casein phosphoprotein (CSN1S1/CSN2) to each of the activated egg lysates as a spiked-in positive control. 4 phosphopeptides from bovine CSN1S1 and CSN2 were identified by both MSA and NL methods. As expected, all of these phosphopeptides showed significant increases in abundance in activated eggs under both control and CanB2 kd samples (Figure

3.2B). However, it was surprising that these phosphopeptides were detected at low levels in mature oocyte samples. This is likely a result of interference introduced by peptide fragments of similar mass-change ratios that were unintentionally co-isolated with the target peptide. Compression of abundance ratios caused by this phenomenon in multiplex isobaric labeling based quantitative proteomic analysis has been reported by multiple studies in recent years (Karp et al 2010, Savitski et al 2013). Our results are consistent with these previous reports, and suggest that the power of the multiplex system to accurately detect the unique presence of peptides in a subset of the samples in a TMT-10plex set is constrained by technical limitations. But nevertheless, the system is efficient at detecting significant changes of abundances among multiple group of samples.

Germline depletion of CanB2 leads to widespread misregulation of protein phosphorylation states both before and after egg activation

To visualize the dynamics of phosphoproteomic changes during egg activation in control and CanB2 kd samples, we created heatmaps basing on the egg-to-oocyte phosphopeptide abundance ratios in control and CanB2 kd samples (Figure 3.3A). Strikingly, two distinct clusters of phosphopeptides that are significantly up and downregulated during egg activation in control appeared to be misregulated in CanB2 kd samples. This is consistent with our previous observation that fewer phosphopeptides were significantly changed in egg activation in CanB2 kd samples. The heatmap visualization of global proteomic changes, on the other hand, showed that the abundance of whole proteins changed moderately during egg activation in

both the control and CanB2 kd samples (Figure 3.3B). We noted that both the proportion of proteins that showed significant protein abundance level changes and the magnitude of these changes were smaller than those to that observed in phosphoproteomic analysis (Figure 3.3A and B). This is consistent with our finding that for more than 80% of the proteins that were significantly phosphoregulated during egg activation in control or CanB2 kd samples, their whole protein level did not change significantly (Figure 3.2A).

Comparison of the lists of up and downregulated phosphopeptides in control vs CanB2 kd samples showed that 74% and 63% of the phosphopeptides whose abundance significantly increased or decreased (respectively) during egg activation in control stayed unchanged in CanB2 kd (Figure 3.3C). There were also peptides that were unchanged in control, but showed significant changes in abundance in CanB2 kd samples (Figure 3.3C). We assigned these different types of misregulations into four categories: Category 1 and 2 comprise phosphopeptides whose abundance significantly increased or decreased (respectively) upon egg activation in control but not in CanB2 kd samples. It is likely that the phosphorylation state changes of the proteins in these categories are mediated by calcineurin or its downstream regulators during the transition. Category 3 and 4 includes phosphopeptides whose abundance significantly increased or decreased (respectively) upon egg activation in CanB2 kd but not in control samples. It is possible that the proteins in category 3 and 4 are kept from significant phosphorylation state changes by calcineurin during egg activation. We noted that Category 2 included three phosphopeptides from Gnu (Figure 3.7A), which is consistent with previous observation of Gnu dephosphorylation upon egg

activation (Hara et al 2016, Lee et al 2003) and the dependence of this dephosphorylation on Calcineurin (Krauchunas et al 2013) (and corroborated with our observation in western blot (Figure S3.3)). Category 2 also included a peptide from YA, a nuclear lamina protein that is known to be dephosphorylate in WT but not in CanB2 kd during egg activation (Krauchunas et al 2013). These findings further validated that our dataset is able to effectively capture the misregulations of oocyte phosphoproteome during egg activation caused by CanB2 germline depletion. Analysis of the proteins in each category using Panther Enrichment analysis algorithm (Mi et al 2017) showed that, category 1,2 and 4 are enriched with regulators of biological processes that are relevant to meiosis and early embryogenesis (Figure 3.4 A B and C), suggesting that components in pathways that regulate different biological processes are likely subjects to different types of phosphoregulations upon egg activation Category 3 includes only 16 proteins and showed no significant enrichment of terms. A combined list of misregulated peptides from MSA and NL datasets can be found in Table S3.2.

Abnormal protein phosphorylation state changes could be a result of aberrant phosphoregulation either during egg activation or before egg activation, in the mature oocyte. Calcineurin has not been reported as essential for oogenesis, and CanB2 KO oocytes (germline clone) arrested at metaphase of meiosis I normally (Takeo et al 2010). We explored whether depletion of CanB2 caused abnormal phosphorylation in mature oocytes. We calculated the phosphopeptide abundance ratio in CanB2 kd oocyte relative to control oocyte for the MSA and NL datasets. Surprisingly, we identified 41 phosphopeptides from 34 proteins (MSA and NL datasets combined)

with significantly altered abundance in CanB2 kd oocytes compare to control oocytes (Figure 3.5), suggesting a role of CanB2 in regulating their phosphorylation state in mature oocytes. This group of protein include regulators of chromatin organization, cell cycle progression, nucleocytoplasmic transport and oogenesis. Intriguingly, this group also included 2 phosphopeptides from Calcineurin catalytic subunit CanA-14F and Pp2B-14D, suggesting that the absence of CanB2 significantly influenced the phosphorylation state of the calcineurin catalytic subunit.

Strikingly, 40 out of the 41 misregulated phosphopeptides were lower in abundance in CanB2 kd oocytes, indicating that CanB2 is required for maintaining normal levels of phosphorylation at the phosphosites on these peptides before egg activation. Since calcineurin is a phosphatase, it is likely that it regulates these sites through one or more kinases effectors. It is also possible that calcineurin maintains the phosphorylation of these sites by antagonizing the activity of another phosphatase in mature oocytes.

It is also intriguing that the only peptide that showed higher level of phosphorylation in CanB2 kd mature oocyte is derived from translational repressor Trailor hitch (Tral), suggesting that calcineurin may be involved in the translational repression of maternal mRNAs in mature oocyte.

In summary, our data showed that calcineurin activity is involved in the regulation of hundreds of phosphosites during egg activation, and unexpectedly may also play active roles in mature oocytes (before egg activation).

Activated eggs with CanB2 knockdown show molecular characteristics of metaphase to anaphase transit.

Loss of CanB2 in the germline causes meiotic cell cycle to stall in anaphase of meiosis I (Takeo et al 2010). To gain further insight into the molecular state of meiotic cell cycle in activated eggs with CanB2 kd, we examined the phosphorylation states and protein abundance changes of several cell cycle regulators in order.

First, we searched for regulators of meiotic cell cycle among the proteins with phosphosites that are misregulated in CanB2 kd. In metaphase of cell cycle, Cyclin-Cdk1 phosphorylates and activates kinase Greatwall (Gwl) (Blake-Hodek et al 2012, Yu et al 2006), which in turn phosphorylate and activates Endosulfine, a competitive inhibitor of Cdk1 antagonist PP2A (Gharbi-Ayachi et al 2010, Mochida et al 2010). Intriguingly, we found that phosphorylation at Cdk1 consensus site T606 on Gwl was significantly downregulated in control but not in CanB2 kd activated eggs, indicating high level of Cdk1 activity in absence of CanB2. Consistent with this, dephosphorylation of Endos, a target of Gwl, was not observed in CanB2 kd activated eggs (Figure 3.6A). As mentioned earlier in the results, another target of Cdk1, Gnu (Hara et al 2017), was also misregulated at several phosphosites in CanB2 kd activated eggs (Figure 3.3D). And these misregulated Gnu phosphosites included 2 predicted CdK1 consensus sites T16 and S170, which provide further evidence for high Cdk1 activity in CanB2 kd activated eggs.

Since Cdk1 activity requires the binding of Cyclins, we examined the protein level of Cyclin B (CycB) and Cyclin B3 (CycB3) in our dataset. At metaphase exit, activation of APC/C leads to the destruction of mitotic cyclins (Chang & Barford 2014,

Holloway et al 1993, Parry & O'Farrell 2001, Yuan & O'Farrell 2015). Thus, if anaphase has initiated in activated eggs with CanB2 kd, we expected to see a decrease in CycB and CycB3 protein levels. In control, the level of CycB3 decreased at the completion of meiosis as expected. But this decrease in abundance is not observed in CanB2 kd eggs. Consistent with this, CycB level also did not significantly decrease in CanB2 activated eggs (Figure 3.7B). These data suggest that cyclins are not destroyed and that anaphase has actually not been successfully initiated in CanB2 kd eggs. We noted that CycB protein level did not significantly decrease in the control either, but instead showed a slight increase. We believe that this is because that most of the control activated eggs had completed meiosis and were arrested in post meiotic interphase. Since the eggs were not fertilized, CycB protein, whose translation was promoted by PNG during egg activation, accumulated in the cytoplasm of the egg (Vardy & Orr-Weaver 2007).

Since the persistence of the cyclins may reflect failed APC/C activation, we further queried the activity of APC/C in CanB2 kd eggs by examining the protein abundance change of APC/C target Matrimony (Mtrm), which is a cell cycle regulator degraded at the onset of anaphase (Whitfield et al 2013). As expected, Mtrm protein level strongly decreased during control egg activation, consistent with previous report by Whitefield et al. However, Mtrm protein level remained unchanged in CanB2 kd activated eggs. This, together with the high level of cyclins in these eggs, suggests that the APC/C may not be activated in CanB2 kd eggs.

APC/C activity requires the binding of coactivator Cdc20. In Drosophila, two Cdc20 family proteins, Cortex (Cort) and Fizzy (Fzy) are involved in meiotic cell cycle

regulation (Pesin & Orr-Weaver 2007, Swan & Schupbach 2007, Whitfield et al 2013). Phosphopeptides from Cort were not detected in our phosphoproteomic data, thus its phosphorylation state changes cannot be assessed. However, we detected five Fzy phosphosites. Our data indicate that phosphorylation state (assessed basing on phosphopeptide abundance) at T78 T82, S113 and S133 changes differently in control and CanB2 kd samples (Figure 3.6C). But only the difference at site T78 and T82 was statistically significant, suggesting potential involvement of calcineurin in the regulation of Fzy T78 and T82 during egg activation. Intriguingly, calcineurin has been shown to be crucial for the dephosphorylation of Fzy/Cdc20 upon egg activation in Xenopus (Mochida & Hunt 2007, Nishiyama et al 2007). Inhibition of calcineurin inhibitor prohibits the Fzy dephosphorylation, and causes delayed degradation of CycB. Our data revealed the possibility that similar mechanism exists in Drosophila and calcineurin regulates the progression of meiosis by modulating Fzy or Cort.

In summary, our data showed molecular evidence that CanB2 kd eggs fail to exit metaphase I with high CdK1 activity and unactivated APC/C.

Curiously, we observed a strong dephosphorylation at Cdk1 T14 and Y15 inhibitory phosphosites in control eggs in comparison to oocyte (Table S3.1). But phosphorylation level at these sites were not significantly changed in CanB2 kd eggs (Table S3.1). T14 and Y15 are highly conserved target sites of Wee1 kinase (Berry & Gould 1996, Den Haese et al 1995, Watanabe et al 1995). In Drosophila, polar bodies generated during meiosis are not expelled from the egg. Instead, they are sequestered to the periphery of the egg and fuse together to form a structure called polar body rosette (Mahowald et al 1983). Since this structure is maintained in a mitosis-like-

arrest in early embryo, it is possible that the dephosphorylation of Cdk1 T14 and Y15 in control activated eggs is related to the formation and maintenance of polar body rosette. However, the reason why high level of Cdk1 inhibitory phosphorylation is detected in metaphase I arrested mature oocytes remains unclear.

Phosphorylation states of translation regulators are perturbed in activated eggs with CanB2 knockdown

Another major event of egg activation is the polyadenylation and translation of many maternally deposited mRNAs (Kronja et al 2014, Tadros et al 2007). To assess the impact of CanB2 knockdown on protein translation, we examined the global proteomic data, and searched for proteins whose abundance levels are misregulated in the CanB2 kd eggs in comparison to the control. Intriguingly, of the 133 proteins whose abundance levels are upregulated during egg activation in control, 61 did not change significantly in CanB2 kd eggs (Table S3.2), suggesting that Calcineurin activity may be required for translation of subsets of proteins during egg activation. Since Gnu dephosphorylation is inhibited in CanB2 kd eggs (Figure 3.7A, Figure S3.2), we suspected that PNG is not activated. PNG is a serine/threonine kinase that regulates the translation of maternal mRNA the progression of mitotic cell cycle in early embryo (Eichhorn et al 2016, Kronja et al 2014, Tadros et al 2003, Elfring et al 1997, Shamanski and Orr-Weaver 1991). PNG becomes active when forming a complex with subunits Plutonium (Plu) and Gnu (Lee et al 2003). The dephosphorylation of Gnu upon egg activation allows it to bind with PNG and Plu and thus activates the complex (Lee et al 2003, Hara et al 2017).

We examined the abundance changes of several proteins whose translation is known to be upregulated by PNG upon egg activation. These proteins include CycB (Vardy and Orr-Weaver 2006), Cyclin A (CycA) (Vardy et al 2009) and Smaug (Smg) (Chen et al 2014, Tadros et al 2007). As expected, we observed that the abundance of CycA and Smg increased in control activated eggs in comparison with mature oocytes. But the protein abundance stayed constant in activated eggs with CanB2 kd (Figure 3.7B), suggesting that translation of these proteins may be stalled or abolished in absence of calcineurin activity. As mentioned earlier, the level of CycB also showed an increase in control activated eggs, but stayed constant in CanB2 kd eggs. This suggests that when CanB2 is depleted, both the degradation of CycB mediated by APC/C and the later translation of CycB mediated by PNG are prohibited in activated eggs. The absence of CycA, Smg and CycB abundance level upregulation reflected by our data is consistent with our hypothesis that PNG is not active in eggs knocked down for CanB2. We validated this hypothesis further by comparing the list of 61 proteins whose abundance upregulations were abolished by CanB2 kd, to a list of 46 proteins that are subject to PNG-mediated translational upregulation published by Kronja et al 2014 (Kronja et al 2014). 7 of the 46 proteins are detected and shown to be significantly upregulated during egg activation in the control. However, all of these proteins showed no significant abundance changes in CanB2 kd eggs, indicating that the abundance upregulation of multiple PNG-dependent proteins during egg activation is abolished in CanB2 kd eggs. This is consistent with inactive PNG kinase.

In addition, several other translation regulators, including NAT1, eIF4G and eIF4B, were misregulated at multiple phosphosites in CanB2 kd activated eggs (Figure 3.7C),

indicating that calcineurin may be involved in several different translation regulation mechanisms. Intriguingly, all these phosphosites are downregulated during control egg activation, suggesting widespread Calcineurin-mediated dephosphorylation of translation regulators during this transition.

Inhibitory phosphorylation on S9 of GSK3β by Akt1 is strongly upregulated in control but not in CanB2 kd eggs

Another interesting finding from the phosphoproteomic data is that in control egg activation, Drosophila GSK3β (Sgg) is strongly phosphorylated at N-terminus residue S9, a inhibitory Akt1 kinase phosphorylation site (Shaw et al 1997). The phosphorylation of GSK3β at S9 during egg activation has been demonstrated using western blot in previous study (Sieber et al 2006), and our result is consistent with this observation. But intriguingly, GSK3β S9 phosphorylation was not observed in CanB2 kd eggs (Figure 3.8), indicating that Akt1 phosphorylation of GSK3β is dependent on Calcineurin activity.

Intriguingly, it has been shown that GSK3β phosphorylate Sra at S215 during egg activation, which is crucial for its function (Takeo et al 2012). Since Sra is known to positively regulate calcineurin activity during egg activation, the potential regulatory relationships between calcineurin, GSK3β and Sra will be an interesting story to explore.

3.5 Conclusion

Phosphoregulation of maternal proteins has been suggested as a major mechanism that drives the transition from oocyte to embryo (Krauchunas et al 2012, Guo et al 2015, Roux et al 2006, Presler et al 2017). Therefore, the activities and regulations of phosphoregulators are of great importance to egg activation and early embryo development. Calcineurin is a highly conserved phosphoregulator that respond to a Ca2+ rise. Thus, it may regulate pathways that coordinate egg activation events. In Drosophila, several previous studies showed the importance of calcineurin for different aspects of egg activation (Krauchunas et al 2013, Takeo et al 2010, Horner et al 2006, Takeo et al 2006), but the molecular consequences of calcineurin activity at this transition still remain unclear.

In this study, we utilized quantitative proteomic methods and presented a comprehensive view of the influence of Calcineurin on global and phosphoproteome during egg activation. Our data revealed widespread misregulation of protein phosphorylation changes in CanB2 depleted eggs. Also, the majority of phosphosites that are differentially regulated during control egg activation are misregulated in CanB2 kd, suggesting Calcineurin as an upstream master regulator of multiple egg activation events that affects the phosphoregulation of numerous phosphosites either directly or indirectly.

Our data also demonstrated that Calcineurin may play active roles in mature oocytes as well. It is intriguing that almost all of the misregulations in mature oocytes are down regulations, indicating that CanB2 is important for maintaining the phosphorylated state of these phosphorylation sites in mature oocytes. Since

Calcineurin is a phosphatase, it is likely that this regulation is achieved through one or multiple kinase effector in the oocytes. It will be of great interest to explore whether and how these phosphorylations are important for the functions of the target proteins. In addition, 2 phosphosites from calcineurin catalytic subunits PP2B-14D and CanA-14F are misregulated in CanB2 kd oocytes. One of these sites matches the Cdk1 consensus recognition site (Songyang et al 1994). Further study of the effects of these phosphorylations on the interactions between calcineurin subunits or vice versa, may yield valuable information on the regulation or calcineurin at the time of egg activation.

In addition to identifying phosphosite misregulations associated with calcineurin depletion, our data also provided an overview of the phosphoproteomic and global proteomic landscape in CanB2 depleted eggs, which shed lights on the cellular state of the arrested CanB2 kd eggs. We found that the state of CanB2 kd eggs resembles that of metaphase I on the molecular level, with high Cdk1 activity and low APC/C activity. Yet, homologous chromosomes are apparently separated basing on the images of the CanB2 kd embryos (Figure S3.1C) (Takeo et al 2010). We observe that no known component of cohesin complex showed significant changes in abundance in control or CanB2 kd eggs relative to the mature oocytes, which is surprising given the segregation of homologous chromosomes observed in CanB2 kd activated eggs. It will be crucial to further study the state of arrest in CanB2 kd embryos using biochemical approaches. It will also be important to investigate and understand the high level of Cdk1 inhibitory phosphorylation detected in metaphase I arrested mature oocytes, and the dephosphorylation of these sites during egg activation.

Another intriguing insight provided by our data is the involvement of calcineurin in the regulation of translation factors during egg activation. In addition to the previously reported role of Calcineurin on Gnu dephosphorylation (and thus the activation of PNG), we found that calcineurin is important for the dephosphorylation of several translation regulators at multiple phosphosites. Further investigation is needed to elucidate whether calcineurin regulates these phosphosites directly and whether the phosphoregulation at these sites modulates the activities of these proteins.

Finally, we showed that an inhibitory phosphosite on GSK3β that is known to be upregulated upon egg activation is dependent on calcineurin activity. Since GSK3β was shown to regulate Sra, a calcineurin regulator during egg activation, it will be intriguing to explore whether calcineurin, GSK3β and Sra form a regulatory loop during this transition.

Table 3.1 Summary of phosphopeptides abundance changes in control and CanB2 kd oocyte and egg samples

		control		CanB2	
	Total phosphopeptides	upregulated	downregulated	upregulated	downregulated
MSA	1472	90	141	45	75
NL	1167	70	112	37	61
combined	1864	117	193	56	110

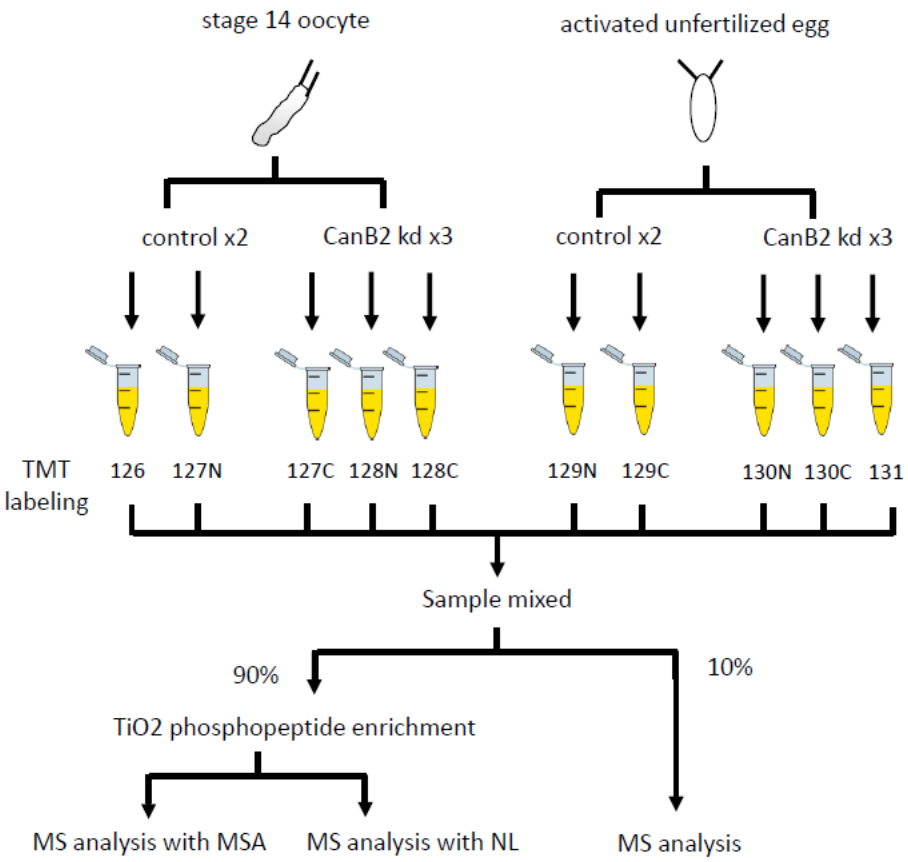

Figure 3.1. Schematics of the experimental set up.

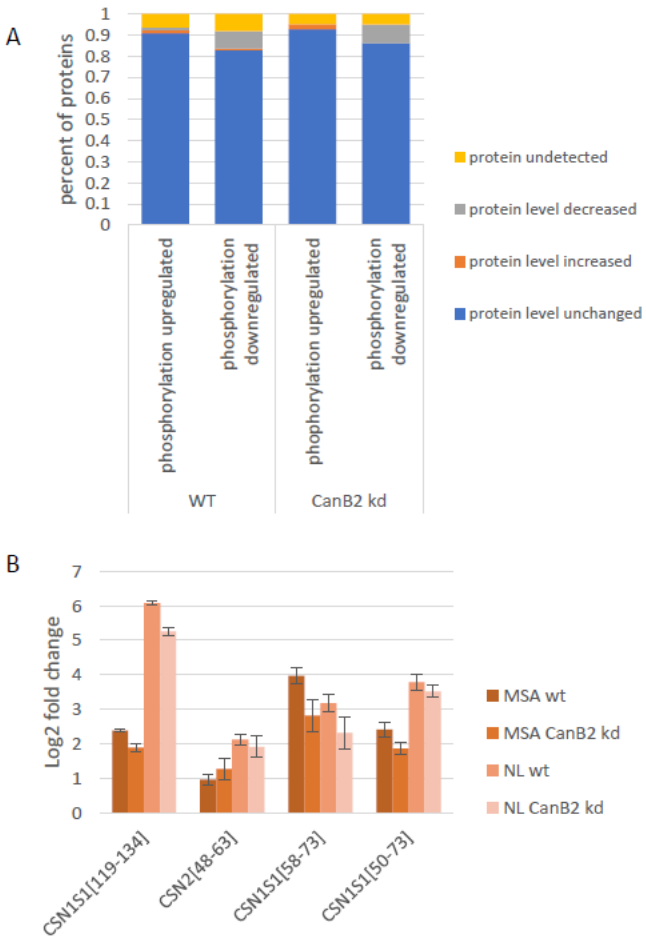

Figure 3.2 Validation of TMT10plex method and samples. (A) The number of proteins with phospho-sites significantly up or down regulated, and the level change of these proteins during egg activation in control and CanB2 kd background. 77 proteins have phosphosites that are more phosphorylated during egg activation in control (70 protein level unchanged, 1 protein level increased, 1 protein level decreased, 5 undetected in global proteomic analysis.); 101 proteins have phosphosites that are less phosphorylated during egg activation in control (122 protein level unchanged, 1 protein level increased, 10 protein level decreased, 10 undetected in global proteomic analysis.); 42 proteins have phosphosites that are more phosphorylated during egg activation in CanB2 kd (38 protein level unchanged, 1 protein level increased, 0 protein level decreased, 3 undetected in global proteomic analysis.); 79 proteins have phosphosites that are less phosphorylated during egg activation in control (68 protein level unchanged, 0 protein level increased, 7 protein level decreased, 4 undetected in global

proteomic analysis.). (B) Phosphopeptide abundance changes of bovine CSN1S1 and CSN2 spiked in positive control.

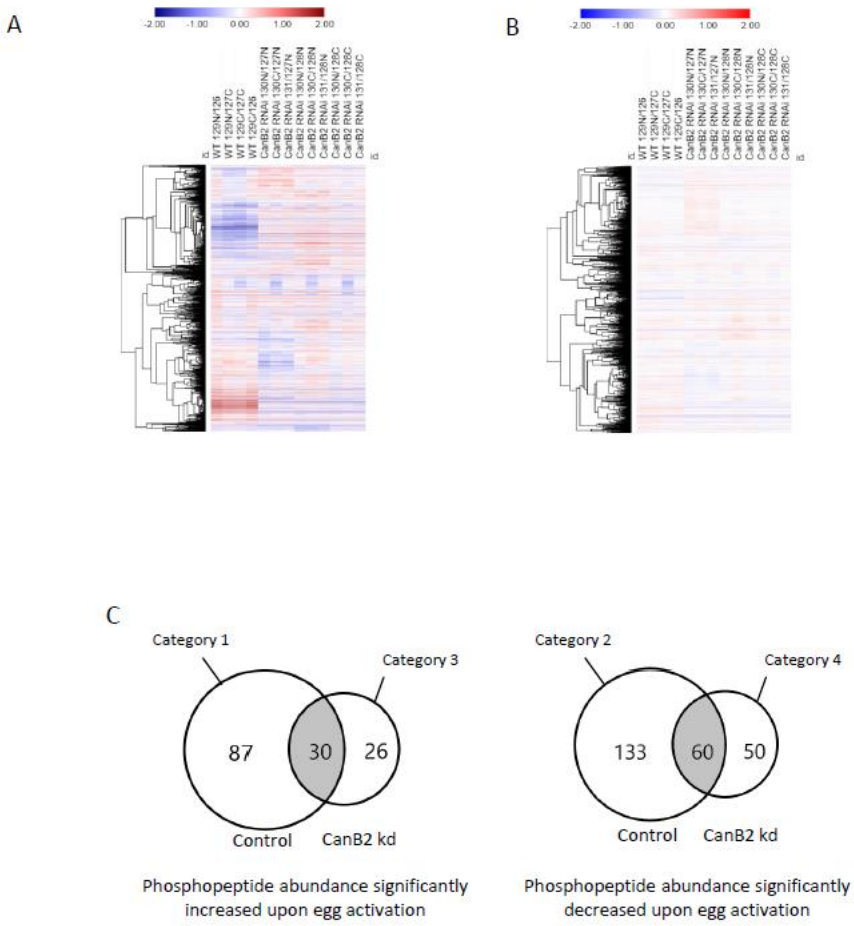

Figure 3.3 Germline depletion of CanB2 caused widespread misregulation of phosphorylation sites during egg activation. (A) A heatmap for the abundance changes of 1998 phospho-peptides during egg activation in control and *CanB2 kd* background. Lane 1-4 show the log2 foldchange of phospho-peptide abundance in control mature stage 14 oocyte vs. control activated but unfertilized egg. The 4 ratios were generated from 2 sets of biological replicates. Lane 5-13 show the the log2 foldchange of phospho-peptide abundance in *CanB2 kd* mature stage 14 oocyte vs. *CanB2 kd* activated but unfertilized egg. The 9 ratios were generated from 3 sets of biological replicates. (B) Similar with (A) but shows the abundance changes of 4039 proteins during egg activation in wt and CanB2 kd background. (C) the overlap of differentially regulated phosphopeptides in control and CanB2 kd egg activation according to both MSA and NL datasets. (C) Comparison of phosphopeptides that are up or downregulated during egg activation between control and CanB2 kd

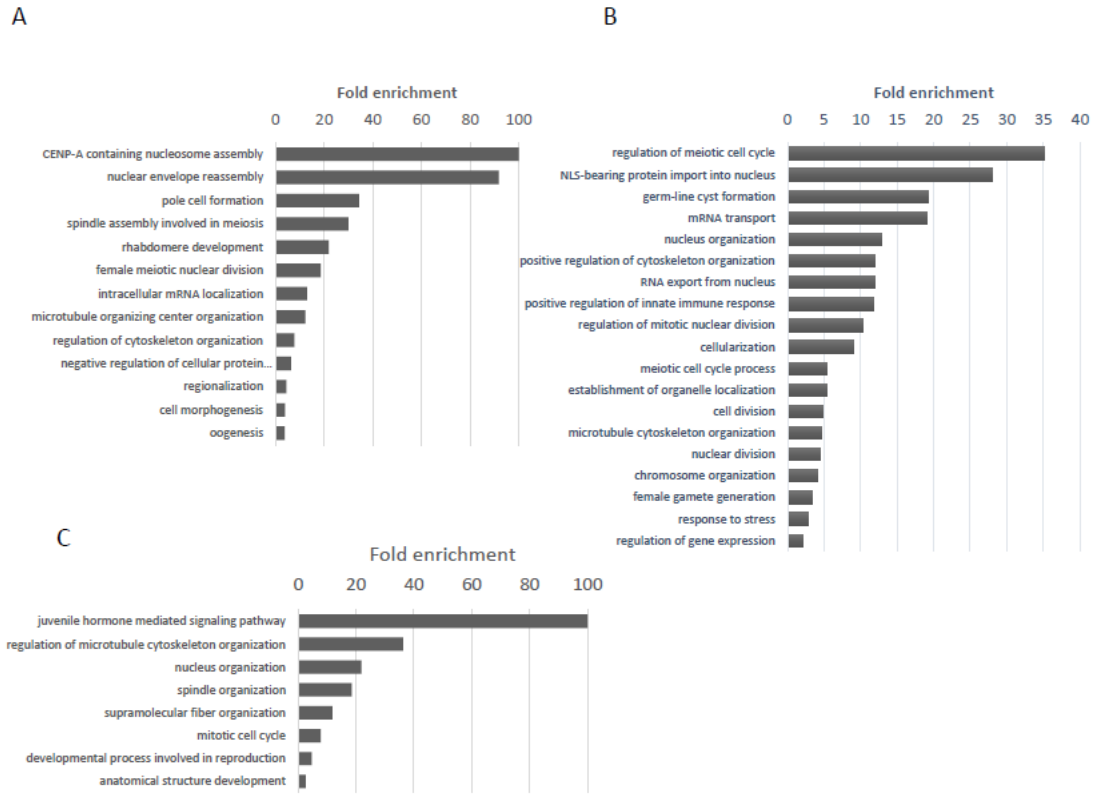

Figure 3.4. Enrichment analysis of proteins with phosphosites misregulated during egg activation under CanB2 kd. (A) Functional classes enriched in proteins that are dephosphorylated upon egg activation in control but unchanged in CanB2 kd (Category 1). (B) Functional classes enriched in proteins that are phosphorylated upon egg activation in control but unchanged in CanB2 kd (Category 2). (C) Functional classes enriched in proteins that are dephosphorylated upon egg activation in CanB2 kd but unchanged in control (Category 4).

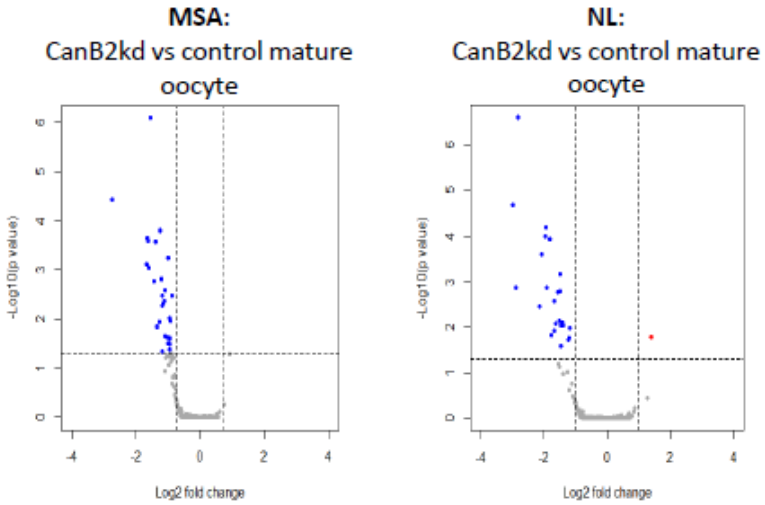

Figure 3.5. Depletion of CanB2 from female germline led to decreased abundance of 40 phosphopeptides from 33 proteins in mature oocyte. Volcano plots of phosphopeptide abundance in CanB2 kd vs wt mature oocytes. X-axis is the phosphopeptide abundance Log2 fold change, Y-axis is the –Log10 of p-value (t-test) against significance threshold (0.74 fold for MSA, 0.99 fold for NL)

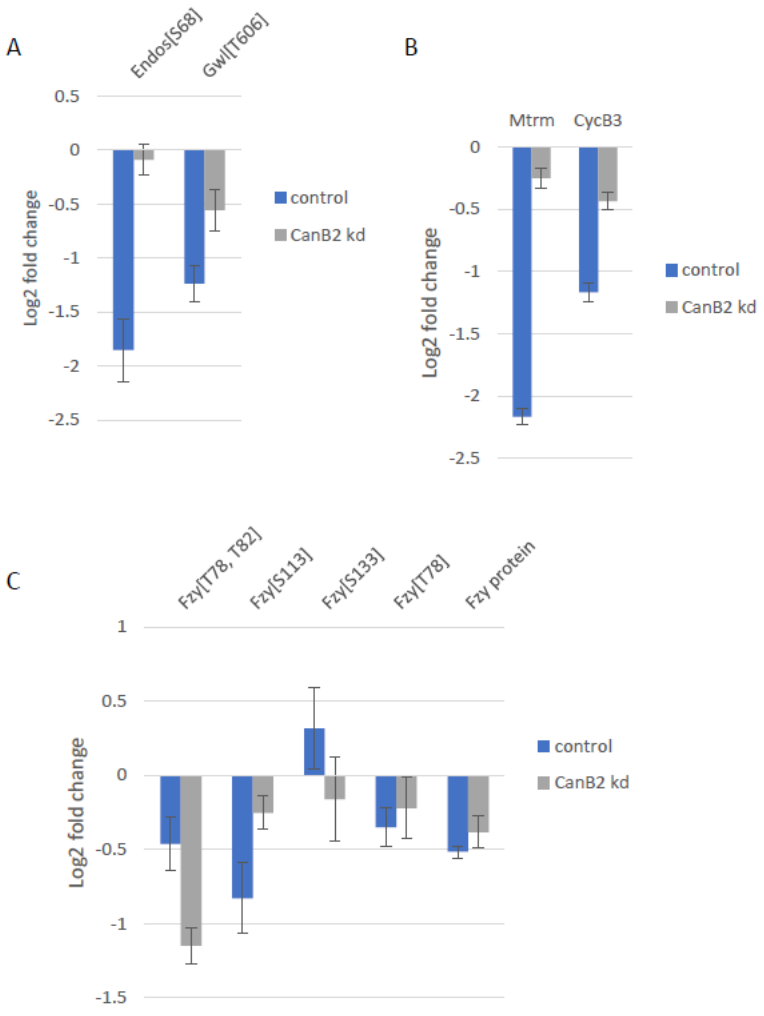

Figure 3.6. Absence of CanB2 led to misregulation of phosphorylation, as well as protein level of cell cycle regulators during egg activation. Y-axis is the log2 fold change of abundance in egg compare to that in oocyte. (A) phosphopeptide abundance change of Endos[S68] and Gwl[T606] during egg activation in control and CanB2 kd. (B) Mtrm and CycB3 protein level change during egg activation in control and CanB2 kd. (C) Abundance of various Fzy phosphopeptides and Fzy protein level during egg activation in control and CanB2 kd.

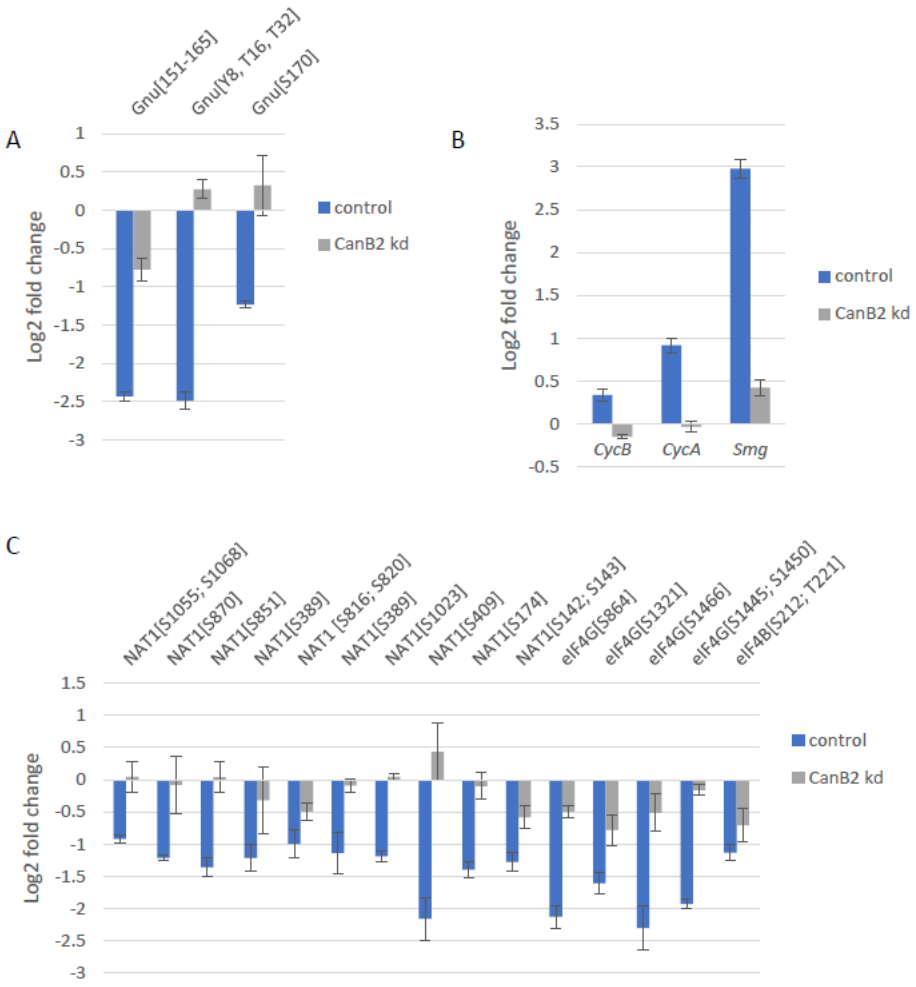

Figure 3.7. Protein translation is disrupted in activated eggs with CanB2 kd. (A) Gnu phosphosites that are dephosphorylated upon egg activation in wt but not CanB2 kd activated eggs. (B) Abundance changes of 3 proteins that are known to be translated upon egg activation under the regulation of PNG. (C) Aberrant phosphregulation were observed at various phosphosites on translation regulators NAT1, eIF4G and eIF4B in CanB2 kd.

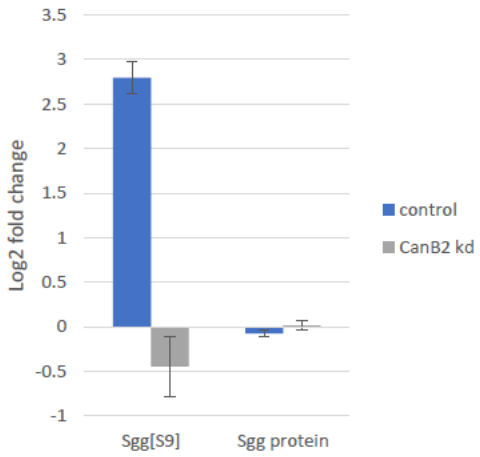

Figure 3.8. Sgg is strongly phosphorylated at at S9 akt1 inhibitory phosphorylation site during egg activation in control but not in CanB2 kd. Y-axis is the log2 fold change of abundance in egg compare to that in oocyte.

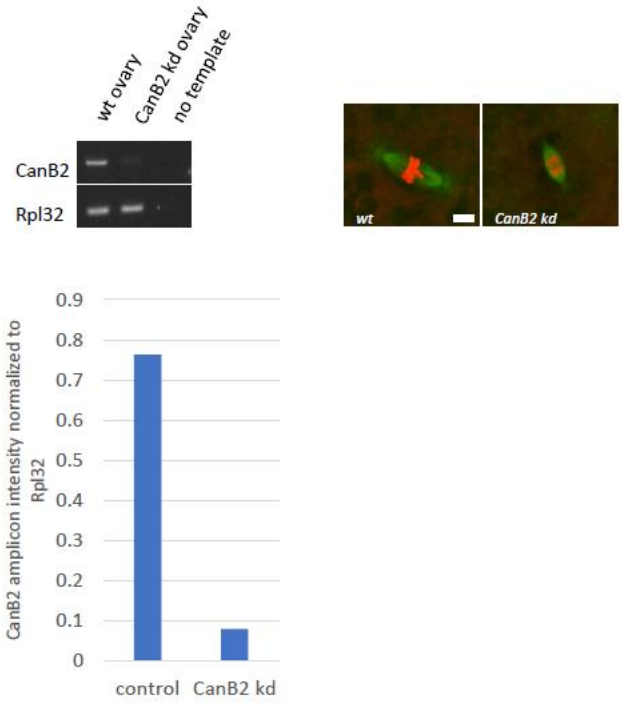

Table S3.1 Germline-specific RNAi efficiently diminished CanB2 expression in the germ cells. (A) RT-PCR gel analysis of CanB2 expression in wt and CanB2 kd ovaries. (B) Quantification of the amplicon band intensity in (A). (C) Spindles in 0.5-1.5hr old fertilized embryos produced by wt and CanB2 germline knockdown females. Tubulin is green, DNA is red. Scale bar = 5µm

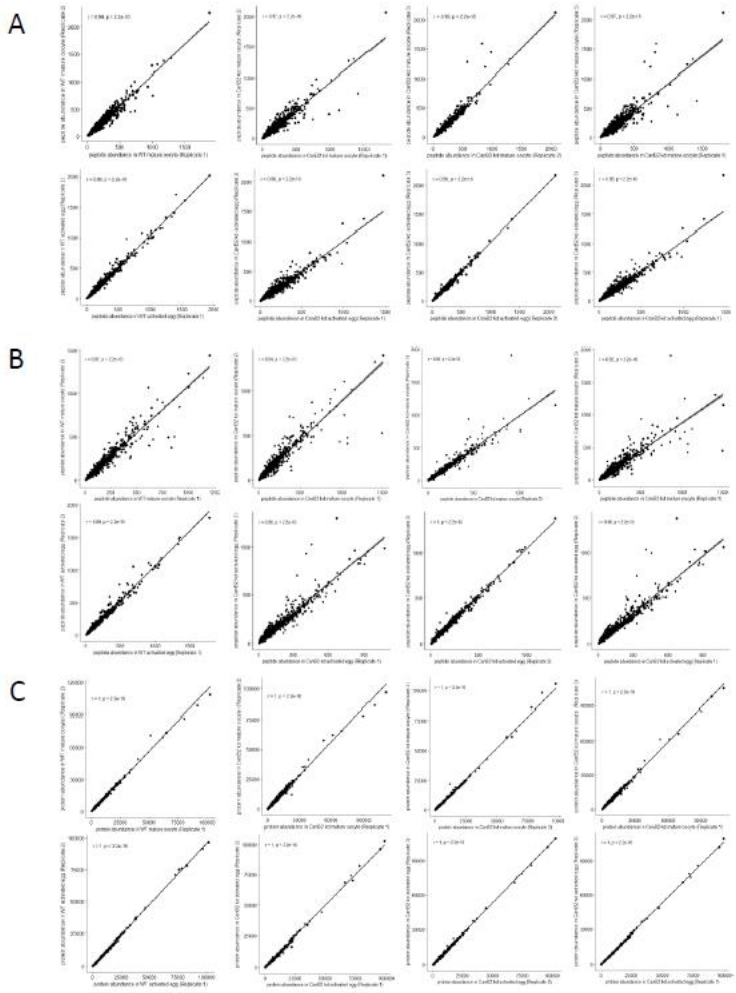

Figure S3.2. TMT10plex Phosphoproteomic and global proteomic analysis generated highly reproducible results. Peptide abundance between biological replicates are highly correlated. (A) Scatterplots of peptide abundance in different biological replicates in MSA dataset. X and Y-axis in each plot represent abundance in two different biological replicates. (B) Similar with (A) but in NL dataset (C) Scatterplots of protein abundance in different biological replicates in global proteomic dataset. X and Y-axis in each plot represent abundance in two different biological replicates.

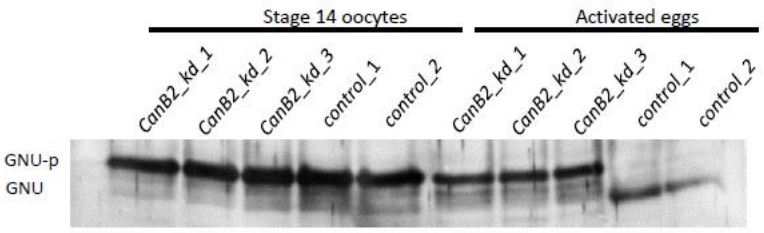

Figure S3.3 Gnu slower mobility form is detected in mature oocyte lysate samples and CanB2 kd activated egg lysate samples, while its faster mobility form is detected in control activated egg lysate samples. Western blot showing GNU mobility in CanB2 kd and control mature stage 14 oocyte samples, and CanB2 kd and control activated but unfertilized egg samples.

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CHAPTER 4

DISCUSSION

Egg activation is a crucial transition through which a mature oocyte completes meiosis and resets its cellular state to support embryogenesis (Reviewed in Chapter One). Though the trigger of egg activation varies among species, a rise of Ca^{2+} in the oocyte's cytoplasm appears to be a deeply conserved initial signal of this transition in all animals studied to date (Sanders & Swann 2016, Sartain & Wolfner 2013).

Egg activation is accompanied by dynamic remodeling of the oocyte proteome through degradation and post-translational regulations. Since transcription is essentially quiescent in oocytes and early embryos (Newport & Kirschner 1982, Zalokar 1976), and translation of new proteins requires time, proteomic changes are likely to be a major driving force of the transition from oocyte to embryo. Among common types of post-translational modifications, phosphoregulation is of particular interest, since it is known to alter protein activity and stability. Moreover, two highly conserved effectors of Ca^{2+} at the time of egg activation, CaMKII and calcineurin, are both phosphoregulators. Recent proteomic studies in Drosophila, sea urchin and Xenopus have reported prevalent protein phosphorylation state changes during egg activation, which also suggest that phosphoregulation is a major mechanism that modulate protein activities during this transition, and proposed the proteins that are actively regulated at this time as valid candidates for important regulators of processes before, during and after egg activation.

The studies presented in this dissertation aimed to shed light on the functions and regulation of proteins that exhibit phosphorylation state changes at the time of egg activation in *Drosophila melanogaster*.

In Chapter Two of this dissertation, I presented a functional screen of maternal protein candidates whose phosphorylation states change during egg activation (Krauchunas et al 2012). I showed that among the 189 candidates screened, 42.8% caused defects in oogenesis, egg activation and embryogenesis when depleted from the germline. These findings contribute to the understanding of egg activation in several ways. First, they showed that a high proportion of phosphoregulated proteins are crucial for different aspects of female fertility, thus validating the functional importance of phosphoproteins at the time of egg activation. Since the screen identified not only factors that are important for egg activation, but also factors that are essential for oogenesis and embryogenesis, it suggests that phosphoregulation is not only a major mechansim that dictates the events of egg activation in response to the upstream Ca2+ signaling, but also a crucial type of modification that facilitate the remodeling of the oocyte proteome to prepare for embryogenesis.

Second, the identification of phosphoregulated proteins that are involved in oogenesis, embryogenesis and different aspects of egg activation revealed many regulators that are not previously known to have roles in these processes. For some of these proteins, their known functions revealed interesting new aspects of the transition from oocyte to embryo. For example, the requirement of PyK for the completion of meiosis suggests that the regulation of glucose metabolism is crucial for meiosis progression. Intriguingly, these newly identified regulators also include new proteins, such as

CG3689 and CG8223, whose functions are unknown in Drosophila, proposing them as interesting subjects for further investigation.

Finally, I showed that though germ cell development is subject to many special regulations, genes that are specialized to function exclusively in female germ cells are quite rare. Instead, vast majority of the genes identified in Chapter Two are ubiquitously expressed and are known to be involved at other developmental stages, suggesting that germ cells employ a very similar set of factors to those in other cell types, and likely achieve their developmental outcomes through special regulatory mechanisms that coordinate the activities of these factors in a different manner. This further emphasizes the importance of understanding the phosphoregulation of these factors during egg activation. In Chapter Three of this dissertation, I worked towards this understanding by dissecting the the activity of phosphoregulator calcineurin upon egg activation with proteomic analysis. I showed that calcineurin influences phosphorylation state changes at more than half of the phosphorylation sites that are regulated during egg activation. I provided molecular evidence that explained why depletion of calcineurin's regulatory subunit led to failure of metaphase exit upon egg activation, and misregulation of abundance changes of more than 90 proteins.

In summary, my studies showed the functional importance of proteins that are subject to phosphoregulation at the time of egg activation, and demonstrated that aspects of the aberrant phenotypes of CanB2 kd eggs can be explained by the misregulation of some of the phosphoproteins. These findings point to the functional importance of phosphoregulation for egg activation. However, further elucidation of how phosphoregulation dictates the transition from oocyte to embryo can only be possible

with genetic and biochemical examination of the effects of phosphorylation on protein activities upon egg activation. With the list of phosphoregulated proteins that are shown to be important for female fertility, and the knowledge of the phosphorylation sites on these proteins, it is possible to generate phosphomimetic and phosphoinhibitory mutants of these proteins and study whether they cause aberrancies during egg activation.

Similarily, for the phosphoproteins that are shown to be misregulated in CanB2 mutant, study of phosphorylation site mutations will also be extremely informative. In particular, my results in Chapter Three indicate that several regulators of meiotic cell cycle (Fzy) and protein translation (NAT1, eIF4G and eIF4B) bear phosphorylation sites that are misregulated in CanB2 mutant. Does the phosphorylation state of these proteins affect their function? Is the misregulation of their phosphorylation state responsible for causing the CanB2 kd phenotype?

Furthermore, identification of direct calcineurin targets will provide valuable information about the signal transduction pathways that connect calcineurin to egg activation events. Our current phosphoproteomic data, by itself, has limited power to detect the direct targets of calcineurin. A great complement to our current dataset will be the phosphoproteomic analysis of oocytes with constitutively activated form of calcineurin catalytic subunit PP2B14D (CnA^{act}). Identification of calcineurin targets may be possible by comparing the two datasets to each other and to WT control, and looking for proteins that are dephosphorylated in control, but not in CanB2 kd activated eggs, and are also hyper dephosphorylated in CnA^{act} mutant oocyte.

Finally, egg activation is a highly conserved process that mark the initiation of life in most if not all species studied to date. The crucial role of phosphoproteins in the regulation of egg activation is evident in multiple species, and is further underlined by the striking discovery of 174 proteins that are phosphoregulated during egg activation in both Drosophila (Krauchunas et al 2012) and sea urchin (Guo et al 2015). By comparing the egg-activation-associated phosphoproteomic changes across different species, and explore the functions of those deeply conserved phosphoproteins, we may be able to uncover essential regulatory pathways that underlay the transition from oocyte to embryo.

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APPENDIX A

TESTING THE EFFICIENCY OF CRISPR/CAS9-MEDIATED GERMLINE-SPECIFIC MUTAGENESIS

A.1 Introduction

In sexually reproducing organisms, the production of gametes is an intricate and highly regulated process. Both the quantity and quality of sperm and eggs are vital for the fertility of the organism. However, study of gene functions in germ cells is constrained by the fact that many of the genes involved in germ cell development are also involved in other biological processes vital for the survival of the organism. For these essential genes, traditional methods of genetic perturbations often result in lethality, preventing the assessment of their roles in the germline.

Three methods have been devised to circumvent this problem. First, chimeric animals can be generated by injecting embryonic germ cells of one genotype into a recipient animal of another (Lee et al 2015). This method can be useful, but still cannot be used to study genes with essential cellular functions. Second, germline clones can be generated by inducing mitotic recombination between homologous chromosomes in the germline, and can create homozygous mutant germ cells in an otherwise heterozygous animal (Griffin et al 2014). This method is primarily used in Drosophila. Its wider application is constrained by the genetic complexity of the animal strains needed.

Third, in several organisms including C. elegans, mouse and Drosophila, germline-targeted RNAi has been used to study gene functions in the germ cells (Ni et al 2011).

Chapter Two of this dissertation demonstrated the use application of this method in Drosophila. However, as we have discussed in the chapter, in vivo RNAi does not always knockdown target gene's expression efficiently, particularly in germ cells, which may lead to uninformative results.

Therefore, it will be beneficial to search for a more efficient and more widely applicable technique to achieve germline-specific perturbation of gene expression in all organisms. The recent method of CRISPR/Cas9-mediated genome editing (Bassett & Liu 2014, Bassett et al 2013, Jinek et al 2012, Yu et al 2013) offers new possibility for the development of such a technique.

CRISPR/Cas9 is a binary system that consists of Cas9, a nuclease that is capable of creating DNA double-strand breaks, and a single guide RNA (sgRNA) that direct the nuclease to specific target site in the genome. This system has the potential to be adapted to perform tissue-specific gene editing (Bassett & Liu 2014, Bassett et al 2013, Jinek et al 2012, Yu et al 2013). In Drosophila, CRISPR/Cas9 has been used to create targeted mutations in the germline with high efficiency (Bassett et al 2013, Gratz et al 2013, Yu et al 2013). However, analysis of gene function in the germline requires mutagenesis to occur early in germline development and work at higher efficiency so homozygous mutations can be created to completely eliminate gene function in the germ cells.

Xue et al 2014 combined CRISPR/Cas9 with the UAS-Gal4 system in Drosophila to create a highly efficient CRISPR/Cas9-mediated conditional mutagenesis (CMCM) system for tissue specific mutagenesis (Xue et al 2014). Intriguingly, with sgRNA expression driven by high efficiency universal promoter CR7T, and Cas9 expression

driven by germline-specific nos-Gal4 driver (Rorth 1998), the authors achieved 60% to 100% mutation rate in the germline for germline genes like *bag of marbles* (*bam*), *centromere identifier* (*cid*) and *nanos* (*nos*) (Xue et al 2014).

To simplify the CMCM system and improve its efficiency, we[6] tested an *in vivo* germline-specific mutagenesis system that was modified from CMCM. The new system differs from CMCM mainly in two ways. First, the Cas9 expression is driven directly by tissue specific regulatory element (nos-Cas9 for germline-specific mutagenesis), rather than through UAS-Gal4 system. Second, the multiple sgRNAs targeting the genes of interest are organized using polycistronic-tRNA-gRNA (PTG) strategy, which is shown to improve the expression of sgRNAs by employing the endogenous tRNA processing systems in human cell (Dong et al 2017). If successful, this system will be a powerful tool for the study of gene function in germline, and can be adapted for use in various organisms.

In this study, we tested the efficiency of this system in germline using 3 genes, *bam*, *cid* and *giant nuclei* (*gnu*). These genes were chosen because their distinct mutant phenotypes. While mutations in *bam* and *cid* abolish gametogenesis (Blower et al 2006, Chen & McKearin 2003, McKearin & Ohlstein 1995, McKearin & Spradling 1990), perturbation of *gnu* in the germ cells leads to maternal effect lethality of the embryos (Freeman & Glover 1987, Renault et al 2003). We designed new sgRNAs targeting each of these genes using new sgRNA evaluation algorithms (Chari et al 2017, Gratz et al 2014, Iseli et al 2007), and drove their expression using strong

[6] A collaboration between the laboratory of Dr. Chun Han, who designed the strategy of germline-specific mutagenesis and created the vectors used in this study. My colleague Qinan Hu designed the sgRNAs that target bam and cid, and performed the sgRNA cloning and stock stabilization.

universal promoters. To optimize the system, we experimented with 2 different promoters (CR7T and U6:3) and evaluated their efficiency in driving the sgRNAs and creating null mutations. We found that the capability of this system to induce mutant phenotype appears to be strongly dependent on the target gene and the design of the sgRNA. We found no substantial differences between the efficiency of the two promoters for sgRNA expression at inducing mutations.

A.2 Materials and Methods

Fly stocks: CR7T-gRNA and nos-Cas9 stocks (kindly provided by Dr. Norbert Perrimon at Harvard University) (Ren et al 2013) were maintained on standard yeast-glucose-agar media, at 23±2°C, under a 12-hr light-dark cycle.

CR7T-gRNA and U6.3-gRNA transgenic stocks: sgRNA sequences targeting *bam* (bam-A: GCAATGAAAACGAAGATCCG bam-B: GTTGCAAGCAATCCAAACCG), *cid* (cid-A: AAAGCAAAACGCGAGCAGCA cid-B: TGCGCAGGGACGCCGGACGG) and *gnu* (gnu-A: TTCGAATGTAAAAGCTTCGG, gnu-B: TTCCTGCCAACGCCTCCAGT, gnu-C: AAAATTAGCAGAAATCCTAC) were designed using Benchling (benchling.com) and evaluated using gRNA Scorer 2.0 (Chari et al 2017) and FlyCRISPR (Gratz et al 2014, Iseli et al 2007) for efficiency and off-target effects. Published sgRNAs for *cid* (cid1cid2, cid1: GGACGCCGGACGGAGGCAGC, cid2: GGAAAGCAAAACGCGAGCAGC) and *bam* (bam1bam2, bam1: GGGCAACGACGACCAGCAGT, bam2: GGAAAGCCACTTGTGAGTACG) were used as published by Xue et al 2014. The sgRNAs were cloned into pAC-CR7T-gRNA (unpublished, created and provided by Dr. Chun Han at Cornell University) or

pAC-U63-gRNA vector (Poe et al 2018). The vectors were sequenced for correct insertion of the gRNA construct. The cloning was performed by Qinan Hu. The gRNA vectors were injected into *w[1118]; nos-phiC31* embryos as a mixture by Rainbow Transgenics.

The injected flies were crossed to *w[1118]* and successful insertions were identified by *mini-white* eye color marker. F1 flies with insertions were sequenced to identify and confirm sgRNA insertion, and 1-2 flies carrying each type of vectors were crossed to *yw; TM3/TM6B* to generate a stable the stock (stabilization of the stock was done by Qinan Hu).

CRISPR/Cas9 mediated conditional knockout: yw; nos-Cas9 AttP2 (BL54591) virgin females were aged 3-5 days and mated to CR7T-gRNA males were aged for 3-5 days. Male and female offspring were collected for the experiment. *yw*; *nos-Cas9 AttP2* males and females were used as control.

Fertility assays

Fertility assays were performed as described in Chapter Two, For each sgRNA set, we tested 10 conditional KO flies and 10 control flies.

A.3 Results

We created stable transgenic stocks that express the new sgRNA set targeting *bam* (refered to as CR7T-bam) and gnu (refered to as CR7T-gnu), the stock with sgRNAs targeting *cid* was during stock stabilization. To offer a positive control for germline-specific mutagenesis, we also created in parallel, transgenic stocks that express the sgRNAs targeting *bam* (referred to as CR7T-bam1bam2) and *cid* (referred to as CR7T-cid1cid2) published by Xue et al 2014.

For the U6:3 promoter, we managed to create stable transgenic stocks that express the new sgRNA set targeting *bam* (referred to as U6:3-bam) , *gnu* (referred to as U6:3-gnu), and *cid* (referred to as U6:3-cid). Similarly, we also created stocks that express the sgRNAs targeting *bam* and *cid* for positive control (referred to as U6:3-bam1bam2 and U6:3-cid1cid2) (Xue et al 2014).

To evaluate the ability of the germline-specific mutagenesis system to induce homozygous null mutation in germ cells, we first tested the fertility of the conditional KO flies. Since *bam* and *cid* are required for gametogenesis in both males and females, we expected that the female flies in which highly efficient editing of *cid* and *bam* created germline-specific homozygous mutations, to have reduced or abolished egg production. Consistent with our expectation, both CR7T-bam/nos-Cas9 females and U6:3-bam/nos-Cas9 females produced severely reduced number of eggs, or no eggs at all. But surprisingly, we did not observe significant reduction of egg production in the females expressing the published sgRNAs against *bam* or *cid,* driven by CR7T or U6:3 promoter (Figure A.1A and B). However, we noted that the egg number of females expressing *bam1bam2* or *cid1cid2* exhibited large variation (Figure A.1A and B), which may render the overall reduction in egg production statistically insignificant. It is possible that the ability of these 2 sgRNA sets to induce null mutation varies among individuals, and work less efficiently in comparison to the new sgRNAs.

To further examine the rate of mutations induced by the germline-specific mutagenesis, we dissected the ovary of the conditional KO females that express sgRNAs against *bam* and observed the tissue under microscope. *bam* mutation is

known to cause distinct accumulation of overproliferated germ cells in the germarium (McKearin and Spradling 1990, McKearin and Ohlstein 1995, Chen and McKearin 2003). Strikingly, over 90% of the ovaries in *CR7T-bam/nos-Cas9* females exhibited the *bam* mutant phenotype in all ovarioles (n = 15) (Figure A.2A and C). *U6:3-bam/nos-Cas9* females also showed high proportion of ovaries with *bam* mutant phenotypes, though more than 20% of the ovaries harbored a mixture of normal ovarioles and mutant ovarioles (n = 21) (Figure A.2A and C). In contrast, females expressing bam1bam2 showed a relatively high proportion of ovaries with no mutant ovarioles (50% in *CR7T-bam1bam2/nos-Cas9,* n = 8; 25% in *U6:3-bam1bam2/nos-Cas9,* n = 24), suggesting that the bam1bam2 sgRNAs failed to induce homozygous null mutations in a relatively large proportion of ovaries.

We next examined the hatchability of the fertilized eggs produced by the conditional KO females (Figure A.1 C and D). Females that express sgRNAs against *gnu* should produce eggs with significantly lower hatching rate in comparison with the control (Figure A.1C and D). We observed that the hatchability of the eggs produced by *CR7T-gnu/nos-Cas9* and *U6:3-gnu/nos-Cas9* was indeed reduced significantly in comparison with the control. We examined the 2-4 hr old embryos for the "giant nuclei" phenotype, and found that ~40% of embryos produced by CR7T-gnu/nos-Cas9 females (n = 52) and ~30% of embryos produced by U6:3-gnu/nos-Cas9 exhibited embryonic nuclei with over-replicated DNA (Figure A.2B and D). This indicates that CRISPR/Cas9-mediated germline-specific mutagenesis is capable of generating homozygous knockout and revealing maternal-effect phenotypes, but our current system may not be efficient enough to cause the phenotype in all embryos.

Finally, we examined the effects of the conditional mutagenesis on male fertility. Most of *CR7T-bam/nos-Cas9* and *U6:3-bam/nos-Cas9* males were sterile (Figure A.3A and B). However, similar to what we observed in the females, mutagenesis with *bam1bam2* and *cid1cid2* did not cause significant reduction of fertility in males either. And surprisingly, *U6:3-cid/nos-Cas9* males also showed no significant reduction of fertility. These results suggest that CRISPR/Cas9-mediated germline-specific mutagenesis via *CR7T-sgRNA/nos-Cas9* or *U6:3-sgRNA/nos-Cas9* is capable of working at high efficiency, but the outcome seems to be highly variable for different target genes, and sgRNA designs.

A.4 Discussion

Our results suggest that both CR7T and U6:3 promoters are capable of driving Cas9-mediated mutagenesis in the germ cells with comparable efficiency. However, the performance of the CRISPR/Cas9-mediated conditional KO system seem to vary drastically with different target genes and different sgRNA designs.

We think that one possible explanation for the differences in mutagenesis efficiency with different target genes may be the temporal relationship of the onset of target gene expression and the onset of Cas9 expression. For genes that are expressed at an earlier timepoint than that of *nos*-driven Cas9 expression in the germline, it is possible that the perdurance of gene products that were made before the Cas9-induced mutation may mask any mutant phenotype. It is also possible that the accessibility of different genes on the genome will affect the efficiency of mutation.

We were surprised to find that the published sgRNAs against *bam* and *cid* did not induce efficient KO in our experiments. It is possible that the UAS-Gal4 system used by Xue et al drives higher levels of Cas9 expression in the germ cells than the *nanos* regulatory element. It is also possible that the tRNA processing machinery behaves differently in Drosophila germ cells in comparison to human cells, and hijacking the machinery may reduce the level of sgRNA expression rather than improving it.

In conclusion, CR7T-sgRNA/nos-Cas9 and U6:3-sgRNA/nos-Cas9 system is capable of highly efficient mutagenesis in the germ cells of Drosophila. With further optimization of the system and careful design of sgRNAs, CRISPR/Cas9 mediated germline-specific mutagenesis may be a valid alternative to RNAi and clonal analysis, and help to reveal previously unobserved gametogenesis and maternal effect phenotypes.

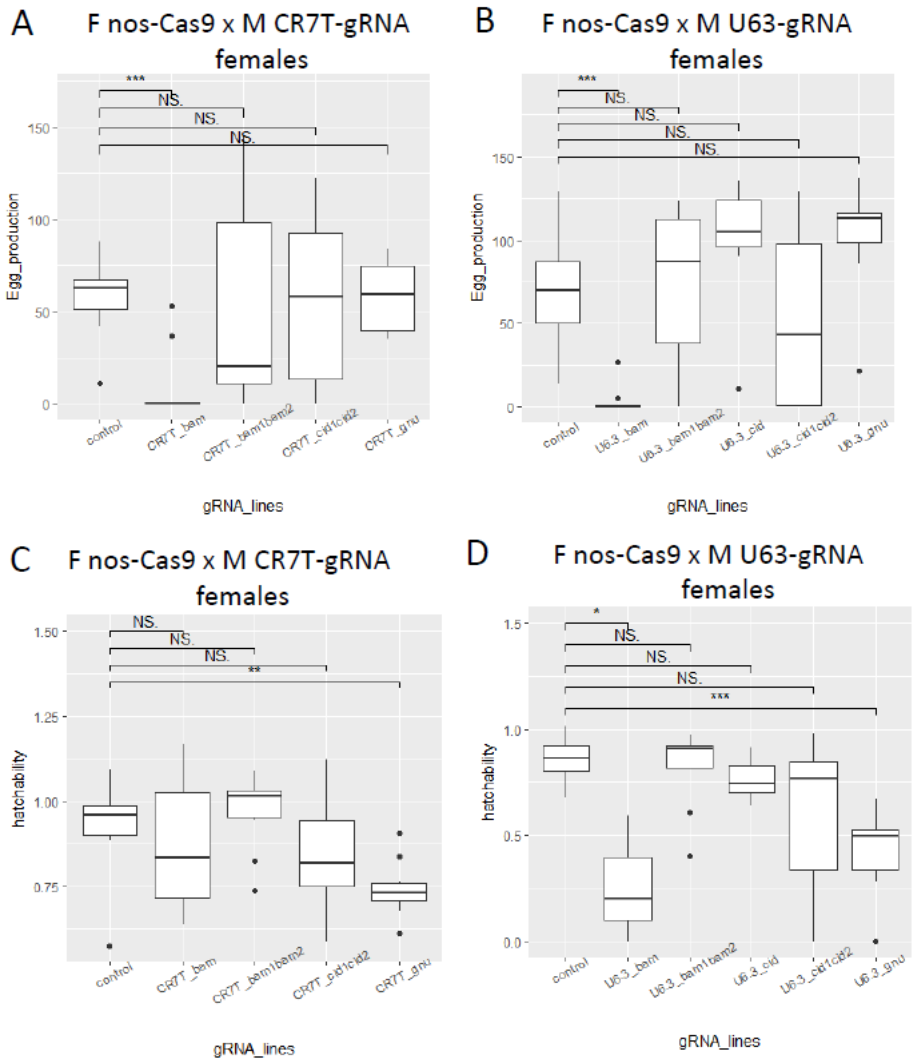

Figure A.1 Germline-specific mutagenesis targeting of bam, cid and gnu had various degrees of impact on female egg production and embryo hatchability. (A) 4-day egg production of females with conditional knockout targeting bam, cid and gnu. sgRNAs were driven by CR7T promoter. The sample sizes, from left to right, are n = 9, n = 9, n = 10, n = 10, n = 10. The egg production between sgRNA lines were compared using t-test. (B) Similar with (A) but sgRNAs were driven by U6:3 promoter. The sample sizes, from left to right, are n = 7, n = 10, n = 10, n = 10, n = 10, n = 10. (C) Similar with (A) but shows the hatchability of the fertilized eggs. (D) Similar with (B) but shows the hatchability of the fertilized eggs.

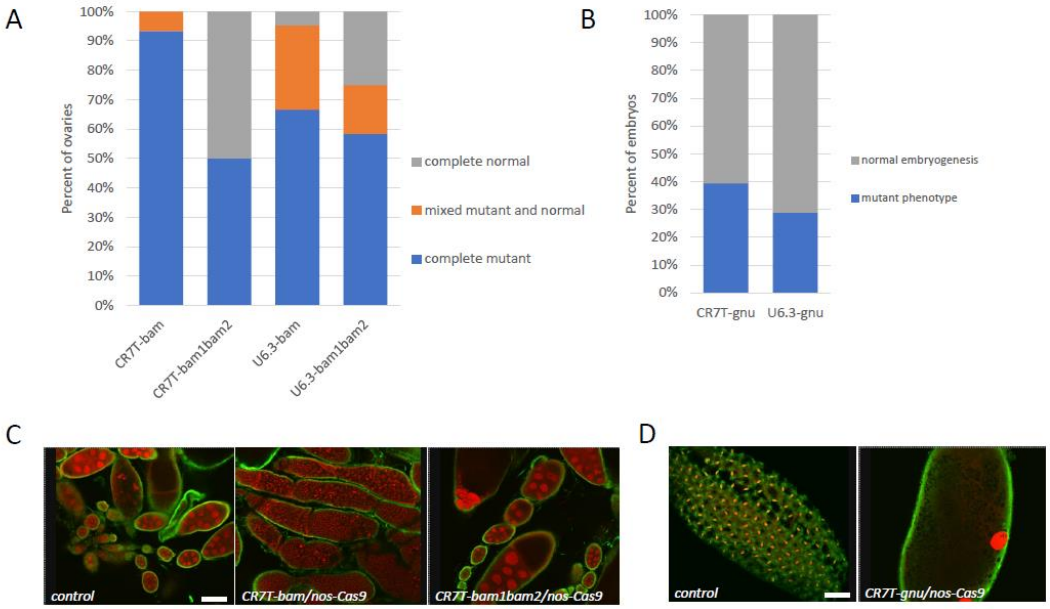

Figure A.2. germline-specific mutagenesis induced expected mutant phenotypes in ovaries and embryos. (A) The proportions of ovaries that showed completely normal, mixed normal and *bam* mutant, and completely *bam* mutant phenotypes in conditional KO females that carry different promoter, bam sgRNA combinations. X-axis shows the promoter-sgRNA combination, Y-axis shows the percentage of ovaries that exhibit each type of phenotypes. (B) The proportions of embryos that showed normal and Gnu maternal effect phenotypes produced by conditional KO females that carry CR7T-gnu and U6:3-gnu sgRNAs. (C) Representative image of ovary from control, *CR7T-bam/nos-Cas9* or *CR7T-bam1bam2/nos-Cas9* females. (D) Representative image of embryo produced by control or CR7T-gnu/nos-Cas9 females.

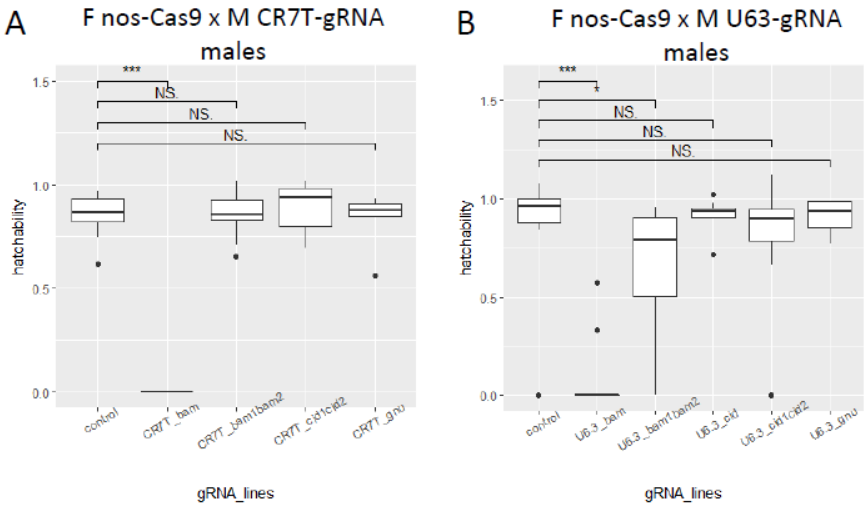

Figure A.3. Germline-specific mutagenesis targeting of bam and cid had various degrees of impact on the fertility of the male conditional KO flies. (A) Hatchability of the eggs produced by ORP2 females mated with conditional KO males that express sgRNA against *bam*, *cid* and *gnu* driven by CR7T promoter. Sample sizes, from left to right, are n = 10, n = 10, n = 9, n = 10, n = 10, n = 10

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APPENDIX B

CREATING MRITYU KNOCKOUT USING CRISPR/CAS9-MEDIATED MUTAGENESIS

B.1 Introduction

In the study presented in Chapter Two of this dissertation, I demonstrated the functional importance of proteins that are phosphoregulated during egg activation. One of these proteins, Mrityu (Mri), is particularly interesting. It is conserved in various species ranging from nematodes to mammals. Yet, its molecular function has not been clarified. Drosophila Mrityu is an ortholog of mouse Btbd10, and is structurally similar to the conserved tetramerization domain of voltage-gated potassium channels.

Krauchunas et al (2012) showed that Mri is likely phosphorylated upon egg activation at 3 sites: S2, T4 and S6, and further demonstrated that the depletion of Mri in female germ cells eliminates the hatchability of the eggs, and causes embryos to arrest early in development, likely in metaphase of the first or second embryonic mitosis (Krauchunas et al 2012). These findings indicate that Mri is essential for early embryogenesis and suggest the importance to further study its function.

In this study, I sought to further dissect the function of maternally provided Mri in early embryos. To do so, I generated a deletion of *mri* using CRISPR/Cas9 and examined the fertility of the mutant females. Surprisingly, in contrast to the phenotype observed in *mri* germline knockdown, *mri KO* females were subfertile, but were

capable of producing viable offspring, which suggests that *mri* RNAi knockdown may have influenced the expression of non-specific targets. Interestingly, the mriKO phenotype cannot be stably maintained for reasons we do not know.

B.2 Materials and Methods

Fly culture and fertility assays: All fly stocks were maintained on standard yeast-glucose-agar media, at 23±2°C, under a 12-hr light cycle. The fertility assays were performed as described in Chapter Two.

Generating mri KO fly stock: Optimized CRISPR target sites at position -254 and 1486 relative to translation start site were selected, and sgRNAs sequence were generated using CRISPR target finder (Gratz et al 2014, Iseli et al 2007). The sgRNA targeting the 5' (AGGAGCCCATCTCCAGCACC)and 3' (GTTGAGCATTACAACGCCCC) of *mri* genomic region were cloned into pU6-BbsI-gRNA plasmid and the homology arms from 5' (1001 bp) and 3' (1022 bp) regions of mri locus were amplified and cloned into the pHD-DsRed-attP plasmid as described on FlyCRISPR website (Gratz et al 2015, Gratz et al 2014). The primers used for 5' homology arm amplification are 5HA_F: GATACACCTGCGACATCGCGCACATAGCACGCTCGTTTA, and 5HA_R: ATCTCACCTGCTTACCTACCCTGGGTGCGCTCAGTCGATA. The primers used for 3' homology arm amplification are 3HA_F: CACTGCTCTTCCTATGCGTTGTAATGCTCAACGAG and 3HA_R: CACTGCTCTTCAGACTACACTCAACGCACGGATTAC. The correct insertion of the sgRNAs and the homology arms were examined using PCR and Sanger's

sequencing (Cornell BRC). The sequencing of sgRNAs and 5' homology arm were performed using M13F primer. The sequencing of 3' homology arm was performed using M13R.

The pU6-BbsI-gRNA plasmids carrying the sgRNAs targeting mri locus and pHD-DsRed-attP plasmid carrying homology arms were injected into *w[1118], Vasa-Cas9* (BL51324) embryos by Rainbow Transgenic Flies Inc.

The injected flies were raised and crossed to *w[1118]; TM3/TM6B flies*. Progeny that expressed DsRed fluorescent marker were selected and mated again *to w1118; TM3/TM6B* flies to generate stable *w[1118]; mriKO/TM3* fly stock. Two separate stocks mri^{KO32} and mri^{KO38} were generated.

Evaluation of off-target: dsRNA targeting *mri* (GL00033) was evaluated using online tool dsCheck (Naito et al 2005). The RT-PCR assessing CG44774 expression was performed using primers CG44774_F; GCAAAGAGAAGCCGGAATTG and CG44774_R; CGTCAGAGTCGCTGTGTTTA.

B.3 Results

Validation of the mutation

To generate a deletion of the entire *mri* locus, I designed sgRNAs targeting 5' and 3' regions of *mri* locus. To facilitate easy identification of flies with the deletion, I created a homology-directed-repair vector that carried 3xp3-DsRed-attP cassette (Gratz et al 2014) flanked by ~1kb homology arms identical to the 5' and 3' genomic region flanking the *mri* locus. In the mutant flies, I expected to find replacement of the mri locus with the 3xp3-DsRed-attP cassette. Thus, as noted in Method, I used DsRed

expression as a marker for successful deletion of mri locus during stabilization of the stock.

To validate the mutation in mri^{KO32} and mri^{KO38} flies, I designed primers that amplify the junction of *mri* locus and its up and downstream region (5' mri and 3' mri), as well as primers that amplify the junction of DsRed and the flanking homology arms (5' DsRed and 3' DsRed) (Figure B.1A). As expected, genomic DNA from the ORP2 control flies showed strong amplification with the 5' mri and 3' mri primer pairs, but no amplification with the 5' DsRed and 3'DsRed primer pairs, while the two KO lines showed the opposite (Figure B.1B). These results indicate that the mri locus is deleted in mri^{KO32} and mri^{KO38} flies. The deletion was further validated with Sanger's sequencing of the mutated locus.

Mri KO females were subfertile

Homozygous mri KO flies are viable and can grow into adults. I thus collected homozygous females from the two KO stocks and evaluated their fertility. Similar to the phenotype observed with *mri* germline-specific RNAi knockdown, the mri^{KO32}/mri^{KO32} and mri^{KO38}/mri^{KO38} females produced normal number of eggs with normal morphological traits. However, the eggs produced by homozygous females showed reduced but not eliminated hatchability (Figure B.2), in contrast to the completely eliminated egg hatchability reported for the *mri* knockdown flies by Krauchunas et al (2012), and validated by my study in Chapter 2. These data suggest that maternally deposited Mri is important but not essential for early development of the embryo. A representative image of 2-4hr embryo produced by *mriKO32/mriKO32* female can be found in Figure B.3.

To explore the possibility that *mri* dsRNA impacted the expression of non-specific targets, I searched for potential non-specific targets of *mri* dsRNA using online off-target evaluation tool dsCheck (Naito et al 2005). According to the software, *CG44774* is a potential non-specific target of *mri* dsRNA. Thus, I examined the expression level of *CG44774* expression in *mri* RNAi ovaries. However, I found no sign of significant reduction in *CG44774* amplicon intensity while mri expression level was reduced to non-detectable level. These results indicate that the expression level of *CG44774* was not affected by *mri* dsRNA.

B.4 Discussion

My results show that *mri KO* females exhibited fertility defects that are less severe in comparison with the mri germline knockdown. There may be several possible explanations to this surprising observation. First, the mri shRNA may have disturbed the expression of one or more unintended target. I have examined the expression of a potential off-target gene CG44774 predicted by off-target prediction algorithm, but did not find significant reduction of its expression in the ovaries of *mri* RNAi females. However, it is still possible that other potential off-target genes may exist but remain unidentified.

Another possibility explanation for our observation is that gene products that are brought by the sperm may be able to rescue the phenotype caused by the lack of maternal products. In the germline-specific RNAi, shRNA in the oocyte may persist after egg activation and continue to mediate destruction of *mri* transcripts and thus lead to more severe hatchability phenotype. This possibility can be tested by mating the KO females to KO males, and observe the hatchability of the resulting embryos.

Finally, a recent study reported that genetic compensations via unknown mechanism can be induced by deleterious mutations in certain loci in zebra fish (Rossi et al 2015).

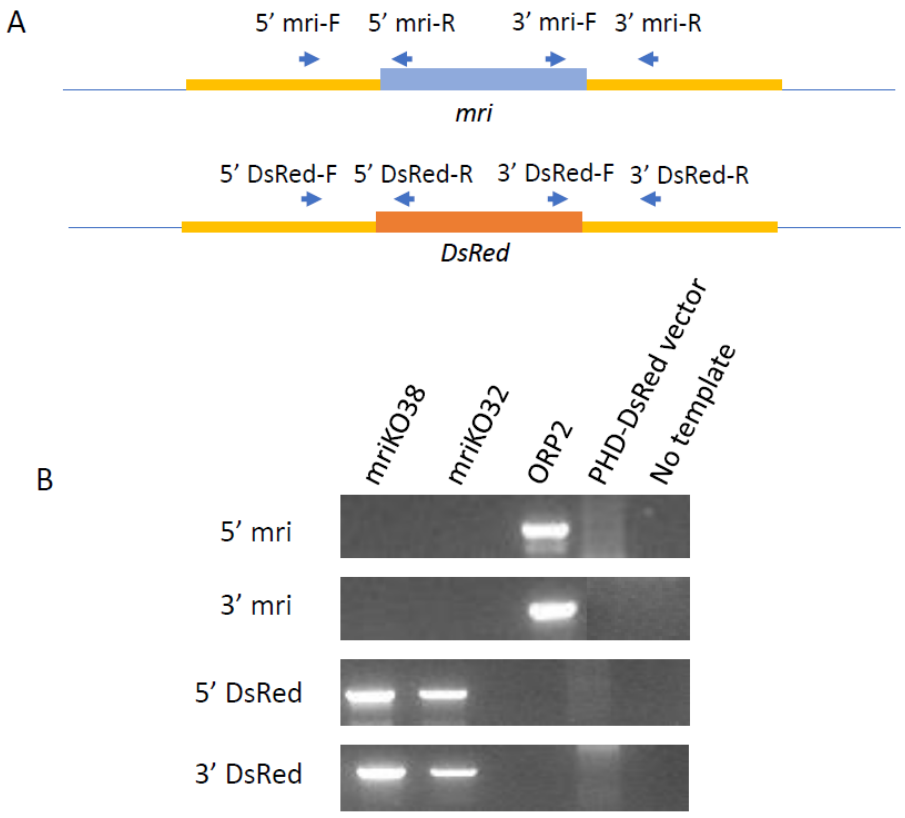

Figure B.1 PCR Validation of the replacement of mri locus with 3xP3-DsRed transgene. (A) Schematic drawings of the annealing sites of 5' mri, 3' mri, 5'DsRed, and 3' DsRed primer pairs in the genomic region. (B) Gel picture of the PCR results with 5' mri, 3' mri, 5'DsRed, and 3' DsRed primer pairs.

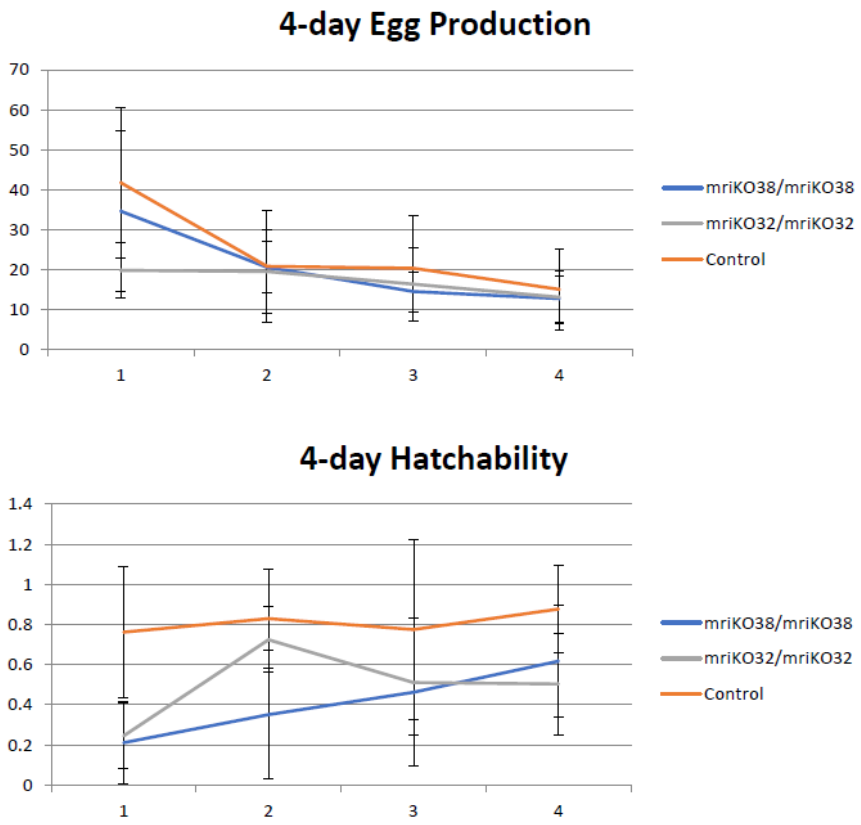

Figure B.2 Comparison of 4-day-egg production and 4-day-egg hatchability between control, *mriKO^{32}/mriKO^{32}* and *mriKO^{38}/mriKO^{38}* females. For 4-day-egg production, X-axis represents day 1 to 4,Y-axis represents egg number. t-test was used to evaluate the difference between *mriKO^{38}/mriKO^{38}* and control (p =0.39), and *mriKO^{32}/mriKO^{32}* and control (p=0.15). For 4-day hatchability, X-axis represents day 1 to 4,Y-axis represents percent of embryos hatched. t-test was used to evaluate the difference between *mriKO^{38}/mriKO^{38}* and control (p =0.0006), and *mriKO^{32}/mriKO^{32}* and control (p=0.017).

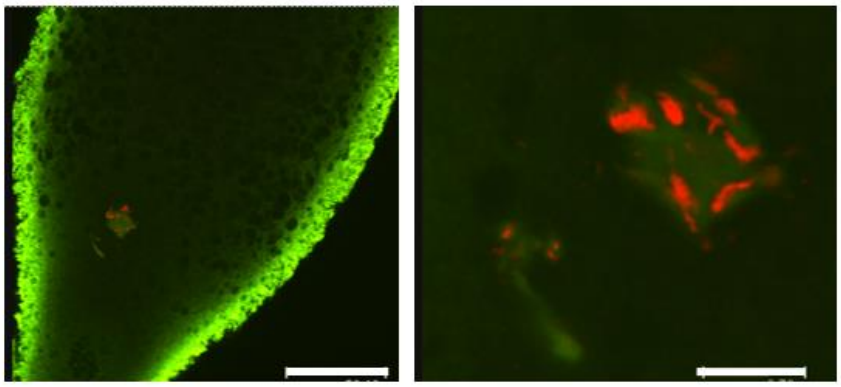

Figure B.3 Early arrested mriKO32 embryos showing abnormal spindle morphology. Green: tubulin, Red: propidium iodide. Scale bar: embryo image 50 µm, spindle 10 µm.

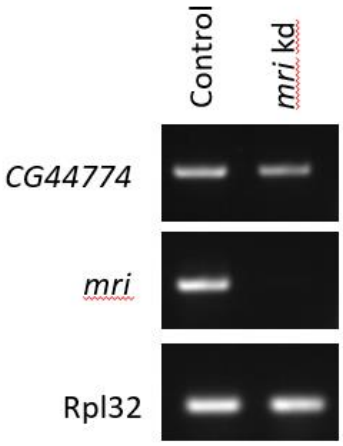

Figure B.4 Expression of *CG44774* was not affected by *mri* dsRNA. RT-PCR showing the expression level of mri, potential mri dsRNA non-specific target CG44774, and loading control Rpl32 in control and *mri* kd fly ovaries.

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