6-21-2018

Signaling Patterns and Genetic Regulation of Drosophila Follicle Rupture

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Ovulation, the liberation of a mature oocyte from its follicle within the ovary, is a requisite step for reproduction, however there is a lack of understanding of the genetic regulation of this process. The goal for this dissertation was to describe the process of ovulation in *Drosophila* to demonstrate the utility for using it as a model to study ovulation. Firstly, the enzyme required for follicular rupture in *Drosophila* was identified as Matrix metalloproteinase 2 (Mmp2). Mmps have been implicated in mammalian ovulation for decades, but this study demonstrated the first genetic evidence of its function. Furthermore, it was shown that after ovulation, the somatic cells that enveloped the oocyte remained in the ovary after ovulation and formed a corpus luteum-like structure, which highlighted the strong resemblance of *Drosophila* ovulation to mammalian ovulation at both cellular and molecular levels. The stimulus for activating Mmp2 activity was identified as octopamine (OA). Through development of an *ex vivo* follicle rupture assay, activation of the OA receptor Oamb (octopamine mushroom body receptor, a G-protein coupled receptor) on mature follicle cells was found to be both essential and sufficient to induce follicle rupture. Further, the signal transduction of the Oamb receptor was described as Gαq/IP3R signaling. Through use of *ex vivo* and *in vivo* calcium imaging, store-operated calcium entry was also demonstrated to be *required* for ovulation. Oamb has been implicated in many physiological and behavioral aspects of Drosophila, but this
was the first description of its signal transduction pathway. Lastly, the coordination of the gain-of competency for a follicle to be able to receive and respond to an ovulation stimulus was investigated. Hindsight, a zinc-finger transcription factor which was specifically expressed in stage-14 follicle cells, was identified as being required for ovulation through the regulation of Mmp2 and Oamb expression. The mammalian homolog to Hindsight, Ras- Responsive Element Binding Protein-1, was able to replace the function of Hindsight in ovulation, further demonstrating the conservation between fly and mammalian ovulation. Altogether, work from this dissertation describes ovulation in *Drosophila* at unprecedented resolution and provides a strong basis for using *Drosophila* as a model for further investigation.
Signaling Patterns and Genetic Regulation of *Drosophila* Follicle Rupture

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B.S., Western New England University, 2013
M.S., University of Connecticut, 2016

A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy at the University of Connecticut 2018
APPROVAL PAGE

Doctor of Philosophy Dissertation

Signaling Patterns and Genetic Regulation of Drosophila Follicle Rupture

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University of Connecticut

2018
Acknowledgements

First and foremost, I am forever grateful that Dr. Jianjun Sun found my application in late April 2013 and reached out to me to join his brand-new lab he was starting at UConn. Choosing to be his first graduate student was one of the best decisions I ever made. JJ- you constantly and forcefully push me into being a better experimenter, presenter, writer, and especially thinker. I appreciate that you never gave up on helping me, despite my occasional inability to hide my Irish stubbornness. Your emphasis on thinking about the logic and story, while still maintaining a critical eye, has really shaped me into the scientist I am today. You are a great role model for your students, as you are a brilliant, enthusiastic, young, hard-working, and successful person. Thank you for everything you do for us.

To the current members of the Sun lab: Liz Knapp, Andrew Beard, Dr. Wei Li, Wei Shen, Dr. Songdou Zhang, Tonya, Jess, Tim, Katya, Audrey, Celina, Taina, Bryan -- I am lucky to have you all as role models and to work with you every day (and surprisingly, yes, I am capable of using your real names). You are as supportive as you are brilliant, as collaborative as you are hardworking, and as collegial as familial. We have become a family, for better or worse, and I wouldn’t have it any other way - right, Wei? To past members of the Sun lab: Shu, Risa, Yaiza, Radhika, Sarah, Jason, Gino, Alison, Halie, Emily, Fiona, Max, Rob, and Iris – you were all such a pleasure to have around and each of you have taught me lesson about working with others and I am lucky to have worked with you for the tenure you were here. I wish nothing but the best for you!

The PNB department has become my family away from my family. I appreciate that everyone has an open-door policy and everyone is so friendly, for the most part. Every lab has contributed, in ideas or reagents, knowingly or unknowingly, to this dissertation. I especially want to thank my committee, Drs. Karen Menuz, Joseph LoTurco, Andrew
Moiseff, and Randall Walikonis for many helpful discussions and support. I would also like to thank Dr. Rahul Kanadia, who is always great to talk to and get advice from. I am grateful for the support from Xinnian Chen, Ed Lechowicz, and Chris O’Connell. Simply by being in her presence, Xinnian makes people strive to be better, and she is such a magnificent role model for a teacher and female scientist. Throughout the years, Ed has been willing to listen to our crazy ideas and make them realistic and tangible. Similarly, Chris has taken our experimental ideas and made them more practical by offering many hours of helping out our lab with imaging dilemmas.

To my friends and family – I would be nowhere without you. My first group of friends was the cohort I entered with: Shanu, Fush, Chris (and Meg, Ellie & Harrison – who may or may not be here when this dissertation is defended!), I love how we all became each other’s biggest cheerleaders, supporters, and sometimes punching bags - through fellowships, papers, weddings, babies, and everything in between. To my Lizard, my best friend. No one understands the past five years the way you do. I can’t put what you mean to me in words (but maybe I could put it into nonsense sounds), and I love you. To Fred & Brittany, you two have become family to me – thank you for accepting my crazy antics and family, and for accepting me into your own families. This also extends to Zach “Uncle Earmuffs”, Megha and Anubhav, Andrew “Princess”, Eric, Anthony and Jake, and Katie – I know we all have a lifetime of excitement ahead of us, and although that may put us at different ends of the country (and maybe sometimes planet!), we’ll never be far away from each other. To my family – Mom, Dad, Sara, (& S.O.’s), thank you for being understanding and supportive when I couldn’t make it somewhere, and for being my biggest cheerleaders, I love you. And there is no way I could make it through the past five years without Josh. I am so grateful to have you in my life as a role model, partner, friend, support beam, and punching bag, despite your “amazingly stupid sleeping habits” (your words, not mine), and I love you.
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CHAPTER 1: INTRODUCTION

UNRESOLVED QUESTIONS IN MAMMALIAN OVULATION

Infertility can be a devastating problem, particularly for those that are unresponsive to infertility treatments. This issue is relatively common, as around 12% of women in America are unable to carry a baby to full-term and give birth (Chandra et al., 2013). Furthermore, it is estimated that >10% of women have seemingly random bouts of anovulation despite having normal oogenesis and hormonal fluctuations (Qublan et al., 2006). This anovulation is typically only detected in women who are undergoing fertility treatments wherein their ovaries are monitored for a stigma indicating ovulation, because normal indicators for an ovulation event, such as a luteinizing hormone (LH) surge, do not always correlate with ovulation (LeMAIRE, 1987). These symptoms (i.e. anovulation despite normal oogenesis and normal hormone levels) characterize luteinized unruptured follicle syndrome (LUFS). Despite the importance of ovulation and its conservation between humans, rats, mice, and fish (Curry and Osteen, 2003; Curry and Smith, 2006; Takahashi et al., 2013), many questions still remain, such as the biological paradox highlighted by LUFS- an instance of a follicle not being completely subservient to the LH surge. Understanding the process of ovulation and follicle rupture is a critical problem to solve and could provide much needed assistance to those couples unresponsive to fertility treatments.

Ovulation, including rupture of a mature oocyte from the ovary, is a requisite step in successful fertilization. Canonically, ovulation is initiated when a surge of luteinizing hormone (LH) is released from the anterior pituitary. LH acts upon the LH receptor (LHR) on the mural granulosa cells of a developing follicle. LHR is a G protein coupled receptor and its activation initiates both the Gαs and Gαq pathways to stimulate ovulation (Breen
et al., 2013). In mammals, there are three critical steps involved in ovulation and they are LH/LHR dependent: oocyte meiosis resumption, cumulus expansion, and follicular rupture. LHR activation on mural granulosa cells induces a suite of signaling cascades to ultimately result in a ruptured follicle, reviewed in (Curry and Smith, 2006). In summary, LHR activation induces increased prostaglandin and progesterone signaling, which in turn induce enzymes such as ADAMTS, plasminogens, and matrix metalloproteinases (MMPs), and it is this coordinated expression and activation that results in spatially and temporally precise follicle rupture. For example, mice that are null for active prostaglandin synthesis (Cox-2 null mice) do not ovulate (Matsumoto et al., 2001). Furthermore, mice that are null for the progesterone receptor are also defective in ovulation (Lydon et al., 1996). Interestingly, when prostaglandin signaling is perturbed, follicle rupture can still occur, however the spatial accuracy is greatly decreased, as ruptured follicles position the oocyte into the ovary instead of into the peritoneal space. When progesterone signaling is perturbed, follicle rupture is nearly completely inhibited. Despite the requirement for prostaglandins and progesterone signaling, the mechanisms and precise site of action in preovulatory follicles remain unknown.

An early hypothesis for how the oocyte ultimately ruptures out of the ovary was that an increase in intraoocyte pressure ultimately “popped” the oocyte out of the ovary at the weakest part of the follicle wall. However, a lot of work has been focused on a different hypothesis: specific temporal and spatial enzymatic degradation, largely through MMPs, of the follicular wall (reviewed in (Liu et al., 2013)) and this is currently the prevailing hypothesis. In 2001, Curry et al. demonstrated dynamic changes in localization via in situ hybridization of Mmps and their inhibitors (tissue inhibitor of metalloproteinase – TIMP) at time points after inducing follicular development (Curry et al., 2001). Because Mmp activity is so highly regulated, they also performed in situ zymography to correlate the expression to activity and found that Mmp activity is localized to the apex of the granulosa cells, or
the area wherein rupture will occur. Nearly fifteen years later, the same group took a comparative approach and investigated expression of Mmps in the rat, macaque, and human (Puttabyatappa et al., 2014). By using immunohistochemistry, western blotting, and qPCR, they identified upregulation of two Mmps, Mmp16 and 14, in all three species examined. Despite the exciting findings that ovulation is conserved across these species, there is an obvious limitation of being able to attribute a function to these dynamic expression profiles to a specific gene.

NEED FOR A MODEL TO STUDY OVULATION

With the devastating number of infertile couples, understanding the mechanisms underlying follicle rupture occurs is essential. This is particularly interesting in terms of the disorder LUFS because, despite hormonal regulation being normal, rupture still does not occur, suggesting a mechanism more complicated than currently understood. A model system is needed to elucidate mechanisms and identify genes necessary for follicle rupture in order to develop therapeutic targets. Some of the experiments and techniques in mouse models are reviewed in: (Liu et al., 2013). In this review, among the concluding remarks, the authors acknowledge the tedious nature of the complexity and redundancy of the genome and the challenges identifying key components. A genetically tractable model with rapid sexual maturation would be ideal to study the process of ovulation. The simplicity of the Drosophila genome, for example only encoding two MMPs, permits research to be done to show direct genetic evidence of some of these implicated enzymes.

Drosophila melanogaster offers the benefits of a simpler genome with fewer redundancies while still maintaining complex physiological processes. The vast genetic tools available, short lifespan, and rapid ovulation of a fruit fly make it an ideal model system to describe processes that occur during follicle rupture and ovulation and also to identify essential genetic components of ovulation. In this introduction chapter, I will
describe the physiological processes involved in ovulation and highlight existing methods that could be used to study ovulation.

**DROSOPHILA ANATOMY AND OOGENESIS**

Female reproductive anatomy is relatively conserved throughout most animals; females have two ovaries (Figure 2.1, 3.1, and 5.1), wherein development of oocytes occurs. The ovaries release the mature oocyte into their respective oviduct (which is the receptacle and transporter for the oocyte, similar to the role of the fallopian tube). The oocyte is transported along the oviduct toward the uterus. This general anatomy and physiology is descriptive of animals from humans to *Drosophila*. Furthermore, similar to human females, *Drosophila* store sperm internally and undergo internal fertilization. Whether an oocyte is fertilized or not, it will be oviposited. In contrast to humans, embryogenesis occurs completely externally in *Drosophila*.

One aspect that is dramatically different between *Drosophila* and humans is the capacity for egg production; *Drosophila* are able to fully develop and lay more than 70 eggs per day at their peak fertility. This enormous reproductive capacity is largely due to their process of oogenesis (see Figure 5.1 for a detailed schematic, and also Figures 2.1 and 3.1). Each *Drosophila* ovary contains approximately 15-20 ovarioles wherein oocyte development occurs. Approximately seven developing follicles (egg chambers) are found within one ovariole and are connected to each other via stalk cells. Similar to a developing mammalian “follicle”, *Drosophila* also have a layer of somatic cells surrounding the developing oocyte. These somatic cells in humans are granulosa and theca cells, wherein in *Drosophila* they are termed “follicle cells”.

Each ovariole is isolated from other ovarioles by an encapsulating ovariole muscle sheath. The group of 15-20 ovarioles per ovary are contained within the peritoneal sheath,
which envelops the entire ovary. At the anterior-most region of the ovariole is a region called the germarium which is composed of a germline stem cells and somatic stem cells which will ultimately give rise to the egg chamber. The germline stem cell divides to produce a 16-cell germline cyst, wherein one of the germ cells is specified as the oocyte and the remaining 15 become the nurse cells, which support the development of the oocyte. A stage one follicle is completely enveloped by a follicle-cell layer and this will bud off, remaining connections via stalk cells to egg chambers posterior- and anterior- to itself. Only after the cyst has developed to 16 cells, the follicle-cell layer migrates to completely enclose the 16-cell cyst. This highly specific cell movement is the first of many these dynamic and complex follicle cells will undergo. Throughout the first six stages of oogenesis, the follicle cells will divide around nine times, to around 800 cells, to maintain coverage of the growing germ cells (King, 1970). During stages six-nine, the follicle cells cease mitosis and undergo endoreplication. During stage nine, the majority of follicle cells move posteriorly to cover the continuously growing oocyte, leaving only ~50 cells (termed "stretch cells") covering the 15 nurse cells. Amazingly, between stage nine and 10, ~6-10 follicle cells at the anterior region detach from the follicle-cell layer and move through the 15 nurse cells to reach the anterior border of the oocyte. These cells will ultimately produce the micropyle (region of the egg chamber that is permeable to sperm). The main-body follicle cell layer continues to cover the growing oocyte between stages 11-14 and undergo gene amplification of genes regulating chorion synthesis. Little is known regarding the fate of the follicle cells after the stage 14 oocyte is ovulated (Spradling, 1993).

Many checkpoints exist to coordinate external cues with this high-energy demanding process (reviewed in (Peterson et al., 2015)). Upon successful completion of the requisite checkpoints, an egg chamber will advance to a preovulatory, stage 14 follicle. Oocytes less mature than stage 14 are not ovulated; the only oocytes to be found outside
the ovary have competed egg maturation (Spradling, 1993) and have been ovulated, suggesting mechanisms to prevent premature follicles from being ovulated. Similar feedback mechanisms may be in place to prevent the follicle-cell layer from entering the oviduct after expulsion of the oocyte, but this has not been investigated.

There has been very little investigation into the stage-14 egg chamber, especially in contrast to all egg chambers stage 10B and younger, and in fact there hasn’t been a consistent way to identify a stage-14 follicle. The canonical review (Spradling, 1993) describes a stage-14 follicle as one that has completed nurse cell degradation and dorsal appendage elongation, however many examples throughout literature depict a stage 14 egg chamber with one or more nurse cell nuclei remaining (For example, figure 1 in (Jia et al., 2016) and figure 2 in (Shravage et al., 2007)). Some groups, as well as chapter 5 in this dissertation, address this inconsistency and attempt to standardize the identification of a stage-14 egg chamber. A 2001 study described two characteristically distinct stage 14 egg chambers in the ovary (Hatsumi et al., 2001). In their description, earlier stage 14 has no nurse cells, a dehydrated main body, and elongated dorsal appendages that maintain very little space between them from the micropyle region all the way to the anterior-most part of the appendages. Later stage 14 egg chambers also have no nurse cells, a “rehydrated” main body, and elongated dorsal appendages, however the appendages are more spread apart at the micropyle region all the way anteriorly. For example, 5-7-day old mated females maintained ~5 early stage 14 and ~0.1 late stage 14 follicles when kept on a “sufficient” nutrient media. Furthermore, they demonstrate that 36% of early stage 14 egg chambers are activated (inactivated oocytes will shrink as their cytoplasm is absorbed by filter paper) whereas 100% of late stage-14 egg chambers are activated. This evidence contradicts recent evidence to show that activation occurs during ovulation and travel through the reproductive tract (Heifetz et al., 2001; Kaneuchi et al.,
Nevertheless, this is an example of an attempt to acknowledge the complexity of a stage-14 egg chamber and demonstrate a physiological significance of its developmental stages.

Successful development of a mature oocyte is the first compulsory phase in female fertility; the oocyte still needs to be ovulated, transported to the uterus, oviposited, and, if fertilized, undergo embryogenesis. These aspects will be described in subsequent pages within this introduction.

**Octopamine Regulation of Fertility**

Many years of work have demonstrated that one imperative signal for ovulation is octopamine (OA), with the first example characterizing the octopamine-null female in 1996 (Monastirioti et al., 1996). OA is synthesized from tyramine through tyramine β-hydroxylase (TβH) and tyramine is synthesized from tyrosine through tyrosine decarboxylase (TDC). Using a null mutant for TβH (TβH<sup>M18</sup> (Monastirioti et al., 1996)), females are unable to synthesize OA, and as a result, are unable to ovulate and experience a dramatic egg-retention phenotype. Females unable to produce their own tyramine (TDC-null) also are sterile with a severe egg-retention phenotype. Interestingly, some reports show that not being able to produce tyramine (TDC-null females) yield an egg-jam phenotype – an egg protrudes out of the ovary and into the oviduct (Cole et al., 2005), but this is challenged in a chapter 3 of this dissertation.

To further investigate this strong phenotype, effort has focused on identifying the receptor and its mode-of-action to induce ovulation. Females that are defective for the OAMB receptor are unable to ovulate (Lee et al., 2003a, 2009). Lee et al 2003 developed several Oamb mutants, wherein they characterized the sterility / egg-retention phenotype. The *in situ* pattern demonstrated the oviduct had the highest expression level of Oamb, along with the spermathecae and the mature follicles of the ovary. To assay ovulation,
they used 3-4 day old virgin females mated 1:1 with wild type males for one hour, then examined the reproductive tract on ice at various time points. By pressing on her abdomen (a method described by (Aigaki et al., 1991)), one would observe an egg ejecting from the ovipositor if a female had just ovulated. The most striking phenotype observed was 12 hours after mating; their mutant alleles were “ovulated” 0-10% compared to their controls at 60%. Described in more detail below, I argue that they were measuring egg transport rather than ovulation.

Follow-up experiments to finding this receptor being responsible for ovulation came from the same group when they sought to determine the tissue in which Oamb was responsible for regulating ovulation. In their 2009 study, they developed a “reproductive-system GAL4” (RS-GAL4), by using a fragment of the Oamb gene, that was specifically expressed in the oviduct epithelium. To determine if the oviduct epithelium was the site of Oamb’s action to regulate ovulation, they used an Oamb mutant (Oamb^{286}) and their RS-GAL4 driver to ectopically express Oamb specifically in the oviduct epithelium. To assay for ovulation, they used 5-7 day old virgins mated 1:3 to wild type males (10 virgins, 30 males / vial) for 3 hours then females were immediately dissected. If there was an egg within the reproductive tract (oviduct or uterus), it was identified as ovulated. Control females “ovulated” around 60% and the mutants “ovulated” around 35%. When they performed a rescue experiment, they successfully reduced the ovulation defect. Females with overexpression of either isoform of Oamb (AS or K3) in the oviduct epithelium in the Oamb^{286} mutant ovulated at levels similar to control (70-60%). These data suggest an essential role for Oamb in the oviduct epithelium to regulate ovulation (Lee et al 2009). However, in chapter 3 of this dissertation, these conclusions will be challenged.

The same group also investigated a possible role for another OA receptor in the oviduct epithelium – Octβ2r (Lim et al., 2014). They determined the Octβ2r mutant was sterile, a phenotype attributable to a lack of ovulation. To assay ovulation, they used 4-5
day old virgins mated 1:3 with wild type males (10 virgins, 30 males / vial) for 18 hours then dissected on ice. If there was an egg within the oviduct (lateral or common) or the uterus, she was recorded as “ovulated”. Wild type females “ovulated” at around 85% whereas Oct2βr mutant females “ovulated” at ~25%, despite showing normal copulation, sperm storage, and post-mating rejection behavior. To determine the site of Octβ2r’s action upon ovulation, they attempted a rescue experiment, wherein they used their previously generated “reproductive-system” GAL4 driver to drive UAS-Octβ2r specifically in the oviduct epithelium— in this fly, the female does not express Octβ2r anywhere except her oviduct epithelium. When they used these females to assay for ovulation, they “ovulated” at levels comparable to control (85-95%), suggesting the site for Octβ2r’s influence in ovulation was also the oviduct epithelium, similarly to their findings with Oamb. When they expressed Oamb (either isoform) or UAS-Octβ3r in the oviduct epithelium to attempt to rescue the Octβ2r mutant phenotype, it significantly increases ovulation rates (~35-50%) from Octβ2r mutant rates (~20%) but does not reach control levels of ~85%, suggesting potential non-overlapping roles of the different OA receptors in ovulation. The model that they currently support is that 1. Octβ2r increases cAMP in the oviduct epithelium to activate PKA and that may induce secretions from the oviduct into the lumen; and 2. Oamb increases [Ca^{2+}]_{i} in the oviduct epithelium, increasing CaMKII activity which could induce nitric oxide signaling to relax the oviduct musculature or to also contribute to luminal secretions.

As mentioned previously, developing egg chambers in the Drosophila ovary are beneath two layers of musculature, which have also been hypothesized to be the site of action of octopamine in regulation of ovulation. The layer closest to the egg chamber is the ovariole muscle sheath (described in (Gutzeit and Haas-Assenbaum, 1991)). Each ovariole has its own muscle sheath enveloping its contents. This layer contains circular bands of muscle fibers that surround the egg chambers, and do not run anteriorly-
posteriorly (Middleton et al., 2006). One layer more distal from the ovariole muscle sheath is the ovarian sheath, or peritoneal sheath, which surrounds the entire ovary. The peritoneal sheath is a large mesh-like network with many gaps that surrounds each ovary (Hudson et al., 2008; Middleton et al., 2006). Both of these muscle networks contain irregular patterns of the thick and thin filaments that are characteristic of “supercontractile” insect visceral muscles, suggesting the ability of these muscles to contract to less than 50% of their resting length. A detailed profile of these muscles is described in (Hudson et al., 2008; Irizarry and Stathopoulos, 2015). The physiology of these ovarian muscles has been described in Middleton et al 2006. They developed a method wherein they can live image ovaries and generate ovariograms based on their movements (indicating a contraction). They found that the musculature of the female reproductive system (ovaries, peritoneal sheath, oviduct, spermathecae) contract rhythmically but independently from each other. They measure peritoneal sheath contractions at the base of the ovary where they remark contractions are most dramatic and easy to measure. Furthermore, they find that the amplitude of these contractions is augmented with OA application, however the frequency remains the same. It is largely because of this experiment that the field has accepted the mechanism of action of octopamine on ovulation is due to musculature changes in both the ovary and the oviduct. However, a peritoneal sheath or ovariole sheath specific GAL4 driver has not been described, so concrete evidence lacks to correlate these muscular contractions to ovulation. Manipulation of the thisbe gene, a ligand for FGF, results in overall disorganization of the musculature of the ovary, and that females mutant for thisbe are sterile; however the precise mechanism in which causes this sterility is unknown (Irizarry and Stathopoulos, 2015).

Other neurotransmitters have been implicated in Drosophila female reproductive physiology. A recent study examined the presence of neurotransmitters before and after mating, specifically to males with and without specific seminal proteins, ((Heifetz et al.,
2014) described below). Trends implicate neurotransmitters such as serotonin, dromyosuppression (DMS), and OA in post-mating responses; however none of the transmitters have clearly been demonstrated to have a role in regulating ovulation, specifically, besides OA. Clearly the orchestration of OA’s action is complex and versatile, and indispensable for regulating fertility, and despite the years of studying this neurohormone, concrete evidence in its regulation of ovulation is lacking.

**Fate of Follicle Cells after Ovulation**

After the mature oocyte is ovulated from the ovary, it is never found with its follicle-cell layer envelopment (Spradling, 1993), suggesting liberation from this layer at some point during ovulation. A 2002 paper examined these follicles in a study about *Drosophila* follicle cells and apoptosis (Nezis et al., 2002). Nezis et al. challenged the assumed hypothesis that stage 14 follicles undergo apoptosis, allowing for them to separate from the oocyte and be ruptured. They examined the most anterior portion of the lateral oviduct – the site where the oocyte would be ruptured into from the ovary. They used three common staining techniques to determine the molecular fate of these cells: acridine orange, propodium iodide, and TUNEL. Acridine orange can detect acidic environments, such as lysosomes and also it has been used to determine the ratio between dsDNA and ssDNA or RNA (it binds nucleic acid and emits green fluorescence when bound to dsDNA and emits red fluorescence when bound to ssDNA or RNA). Propodium iodide is incorporated into condensed chromatin of usually dead cells. And lastly, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) was used to label fragmented DNA characteristic of apoptotic cells. They found that the entry of the lateral oviduct (i.e. the calyx), had accumulated these leftover follicle cells, was positive for both acridine orange and propodium iodide. They interpret these results as these cells undergoing autophagy. However, they are unable to detect TUNEL-positive cells in stage
14 follicles, even when they treat the whole ovary with etoposide, an apoptosis-inducer. This important study characterizes follicle cells that separate from the oocyte during ovulation remain in the ovary. Chapter 2 addresses the fate of the follicle cells after ovulation in greater depth.

**EGG TRANSPORT THROUGH THE REPRODUCTIVE SYSTEM**

The environment in which an oocyte is ovulated into influences whether it will be ovulated or not. The egg is first accepted from the ovary into the lateral oviduct, then is promptly shuttled to the common oviduct, before pausing in the uterus prior to oviposition. The lateral and common oviduct muscles are typical insect visceral muscles that contain skeletal and smooth muscle characteristics: there are striations, and they have slow, rhythmic contractions (Middleton et al., 2006). The oviduct muscles circularly envelop the oviduct lumen. The oviduct epithelium, which surrounds the lumen, has microvilli that likely assist in oocyte transport through secretions (Middleton et al., 2006). Uterine musculature is much more intensive than the oviduct but structurally similar (Middleton et al., 2006). The innervation of these components of the reproductive tract are well characterized, as described below. Approximately 70 times per day, the oviduct must correctly coordinate the previously described events for transporting large oocytes all the way from the ovary to the uterus, positioning them correctly, and preparing them for fertilization.

Proper tonus of the oviduct musculature is essential for an egg to be able to pass through; if the circular oviduct is tightly contracted, the egg will not be able to be ovulated. The role that neuromodulators, such as octopamine, have on the muscle tonus on the oviduct has been intensively studied. Middleton and colleagues (2006) characterized the innervation of the female reproductive tract: nerves branch from the abdominal median nerve trunk to intensively innervate the female reproductive tract, all of which are TDC2 positive. Post-mating increases in octopamine have been demonstrated to be essential in
relaxing the oviduct musculature (Rubinstein and Wolfner, 2013), a process that is dependent on ovulin. Data in support of this claim come from measuring sarcomere lengths after mating in response to increased OA after mating.

In contrast to OA acting directly upon oviduct muscle, there is a body of evidence suggesting OA receptors on the oviduct epithelium, which lies between the oviduct muscle and the oviduct lumen, regulate oviduct muscle. From these studies, Oamb and Oct2βR on the oviduct epithelium activate Ca^{2+} and cAMP pathways, respectively. Their model describes these intracellular pathways could increase nitric oxide signaling from the epithelium to signal to the oviduct muscle to stimulate relaxation (Lee et al., 2009; Lim et al., 2014), described previously). The common oviduct is intensively innervated by TDC2-positive neurons and some of these neurons are also glutamatergic. Using immunohistochemistry to identify neurotransmitter accumulation at synaptic boutons, one group was able to associate how different neuromodulators were released over time in response to mating (Heifetz et al., 2014). They found the major neurotransmitter within the oviduct is octopamine; however they also found there is also a considerable amount of serotonin activity (Heifetz et al., 2014). Currently, it is unclear what the role of serotonin is in regulating the oviduct. Nevertheless, the relatively narrow oviduct undergoes many morphological changes to allow for ovulation to occur and for the oocyte to be transported to the uterus.

Mating induces many changes to the physiology of a female, specifically the transfer of seminal fluid proteins, for example: increased ovulation, egg production, and reduced re-mating. Recent work from the Wolfner lab described a new method they developed using high-resolution, multiscale micro-computed tomography (micro-CT) scans to visualize the entire Drosophila female and make observations about changes that occur after mating (Mattei et al., 2015). They froze females that were either currently mating in copula, at 10 min after the start of mating (ASM), or at 35 min, 90 min, 3 h, 5 h,
and 8 h ASM. Among interesting findings, such as the vaginal teeth of a female interlock with the male claspers during mating, they were able to demonstrate the increased, then decreased amount of space that the ovaries take up within the abdomen. In virgin females throughout ~35 min ASM, the ovaries constitute most of the abdominal cavity, which is indicative of egg retention. After 8 h ASM, there is a reduction in ovary size, correlating with a lack of mature stage 14 egg chambers. This resolution shows the rapid maturation of immature egg chambers and release of those mature egg chambers within the first 5 hours ASM, and the slowing down of this process 8 hours ASM. Interestingly, they found the reproductive tract has a counterclockwise coil in virgin females throughout 90 min ASM. However, between 90 min ASM and 5 h ASM, this coil straightens out, until 8 h ASM where it appears to have re-coiled, a process they have experimentally attributed to the transfer of ovulin in the seminal fluid.

Although most research involving the oviduct-ovulation relationship has been focused upon the oviduct muscles, the epithelium also maintains important roles for regulating egg transport. Secretions from the secretory glands of the spermathecae and parovaria into the lumen are also essential for proper ovulation. Using a lozenge knockout fly to eliminate reproductive glands, (Sun and Spradling, 2013) a significant impact of secretory cells on ovulation was observed. To confirm this mechanism, the authors knocked down secretory cells specifically within the reproductive tract (dpr5-Gal4) and were able to correlate the efficiency of ovulation to the number of properly formed secretory cells (Sun and Spradling, 2013). This new evidence directly implicates communication between the products that are secreted within the oviduct and the ovary to coordinate ovulation.

**OVIPOSITION**

After an oocyte has been matured, ovulated, and transported, it can finally be laid.
Females can retain eggs in their uterus for extended periods of time until they find an appropriate substrate to lay their egg on. The time an egg spends in the uterus can be estimated by using the following formula: \[
\text{time to lay one egg (time given/total eggs laid)} = \text{time in uterus (time to lay one egg * percent of eggs found within the uterus) + time to ovulate (time to lay one egg – time in uterus)} \] (Sun and Spradling, 2013). This method is an estimate and relies on an experiment wherein females are dissected and the location of an oocyte within the oviduct is recorded. Using this estimation, transport through the oviduct is almost instantaneous, whereas females can hold an egg in their uterus for ~10 minutes.

If the egg has been successfully fertilized, embryogenesis will occur externally. For a species to propagate, it is essential its embryos are viable; therefore, an egg must be laid in an optimal environment in which the offspring will thrive. A 2008 study from the Jan labs characterized this process in detail (Yang et al., 2008). *Drosophila* females go through a very stereotyped behavior that precedes oviposition (“Ovipositor motor program”): 1. immediately prior to oviposition, a female will extend the posterior-most part of her abdomen so her ovipositor is contacting the egg-laying substrate, then she will lay the egg; 2. Cleaning and Resting – the female will touch her ovipositor with her hind legs as if to clean it, then remain immobile for a few seconds; 3. Searching – after the female has rested, she will search for another appropriate site to lay an egg, to repeat these stereotyped steps (Yang et al., 2008). To determine the circuitry of the ovipositor motor program, they identified a subset of ILP8 neurons (similar to mammalian relaxin, responsible for widening the cervix for birth) that are expressed in the reproductive tract near the uterus. Hyperpolarizing these neurons resulted in abolition of the ovipositor motor program, and thus no egg laying. Furthermore, silencing of ILP8-neurons resulted in an “egg jamming” phenotype; eggs were found in the lateral oviduct and the common oviduct, a highly uncommon phenotype (Yang et al., 2008).
The preferred substrates that a female will choose to lay her eggs are well characterized. *Drosophila* have been found to be attracted to a substrate with acetic acid (AA) to lay their eggs (Gou et al., 2014; Joseph et al., 2009; Yang et al., 2008). AA is a natural byproduct of fermenting fruit, a preferred food choice of fruit flies. Females prefer to oviposit their eggs on a substrate containing AA, a behavior mediated by the gustatory system (Joseph et al., 2009). Using *pox-neuro* mutants (homozygous *poxn^{AM22-B5}*), to transform taste bristles into mechanosensory bristles without gustatory receptors, they found that females reduced their preference for AA-containing substrates. Despite having AA as a preferred substrate to oviposit, females also exhibit positional avoidance toward it, a behavior mediated by primary olfactory organs (Joseph et al., 2009). Surgical removal of the primary olfactory organs, 3rd antennal segments reversed their positional avoidance of AA-containing substrates (Joseph et al., 2009). Further studies into the neuronal circuitry of the preferences to oviposit on AA-containing substrates uncovered an interesting set of reproductive tract neurons. Gou and colleagues (2014) found that it is the egg within the reproductive tract that drives female behavior to go towards the AA-containing substrate to oviposit the egg (Gou et al., 2014). This group found that having an egg jammed within the reproductive tract was sufficient to guide a female toward the AA-containing substrate. They investigated the potential for ppk* neurons within the reproductive tract to be mechanosensors. By reducing activity of ppk* neurons, the females still had eggs jammed within their reproductive tract, however they didn't exhibit the AA preference that was typical of an “egg-jam” phenotype. They concluded that it is these ppk* neurons within the reproductive tract that sense an egg present and relay that signal to other processing centers within the VNC or CNS to direct them toward the AA-containing substrate to oviposit the egg (Gou et al., 2014).

Further studies into *Drosophila* oviposition preference determined that a female will prefer to lay an egg on softer substrate rather than a hard substrate and a plain
substrate to a bitter (lobeline) substrate (Yang et al., 2008). Females will feed from a sucrose-containing substrate, but avoid laying their eggs on it in a dose-dependent manner (Yang et al., 2008). Authors have attributed the preferences characterized above to a subset of sweet taste receptor neurons (Gr5a). When Gr5a neuronal activity was reduced by overexpressing hyperpolarizing Kir2.1, females reduced their avoidance to the sucrose-containing substrate (Yang et al., 2008). This avoidance is reduced when the sucrose-containing substrate becomes too far away from other options, suggesting there is a decision based upon effort – is it worth it to travel further to lay eggs for that substrate? Alternatively, it is possible that the females are exhibiting a behavior to prevent the first-instar larvae from hatching from the embryo and having to travel too far for a sucrose-containing food course. A follow-up project from the Heberlein lab’s 2009 paper (Joseph et al., 2009) further investigated the simultaneous avoidance/attractant behavior to lobeline (Joseph and Heberlein, 2012). They determined that Gr66a, a gustatory receptor, is necessary for both the situational avoidance to lobeline and also necessary for the egg laying attraction (Joseph and Heberlein, 2012).

Another preferred oviposition substrate for female fruit flies is ethanol, another byproduct of fermenting, rotten fruit. Unlike the AA-containing substrate preference, females do not actively avoid ethanol-containing substrates (Azanchi et al., 2013; van Delden and Kamping, 1990; Richmond and Gerking, 1979; Siegal and Hartl, 1999). Using a similar approach to the AA-preference study, researchers removed the third antennal segment and found that the preference for ethanol-containing media was not eliminated, suggesting that ovipositing females are attracted through different sensory input than their antennae (Azanchi et al., 2013). They also used the *pox neuro* mutants to render their gustatory spines as mechanosensory spines and this manipulation made females avoid ethanol-containing oviposition sites, directly implicating the gustatory system in ethanol-sensing substrates. They further determined the circuitry regulating the preference for
ethanol-containing substrates as an oviposition site, and they found dopaminergic neurons as a likely candidate. They silenced a subset of dopaminergic neurons (PAM neurons) by using tetanus toxin (UAS-TeTx) and showed a decreased response to ethanol, suggesting these are the subset of neurons regulating ethanol preference (Azanchi et al., 2013).

Oviposition and ovulation are tightly related. An egg needs to be ovulated before it can be oviposited. Further, because a female will hold onto an egg until it finds a preferred substrate, it would be wasteful for a female to ovulate before the next egg has been laid. A feedback mechanism must occur between the uterus having an egg in it and the ovary to prevent another from being ovulated. Data from Yang et al 2008 suggest the possibility that ILP8-neurons are not only responsible for the substrate selection for egg laying, but also a potential role in relaying an inhibitory signal to the ovaries to prevent ovulation. If a female is choosing to not lay eggs and they are getting built up in her reproductive tract, in contrast to not being ovulated and just remaining in the ovaries, there must be a lack of feedback in the hyperpolarized ILP8-neurons in contrast to control conditions. It seems unlikely that the feedback mechanism would come from the ppk+ mechanosensitive neurons, because the phenotype of silencing those neurons involves only one egg being present within the oviduct (Gou et al., 2014). Deciphering this phenotype becomes tricky experimentally – depending on the method of anesthesia, one could artificially induce ovulation and therefore increase the presence of eggs within the reproductive tract. For example, anesthesia with CO2 increases the presence of eggs within the lateral oviducts. Another common mode of anesthesia is Fly Nap, which researchers have used to induce ovulation experimentally (Kaneuchi et al., 2015). Nevertheless, this potential feedback mechanism is currently unknown but would be very interesting to uncover.
NEED FOR A METHOD TO STUDY OVULATION

Many systems influence the process of ovulation as described above, so it is imperative to consider multiple aspects when characterizing an ovulation phenotype. Firstly, a strict and clear definition of ovulation and follicle rupture must be standardized. The following scenario will help resolve the differences between ovulation and follicle rupture: consider if an ovum is ovulated from its somatic follicle cells into the ovary, it has not been ovulated; rather it has been ruptured improperly. Therefore, I would suggest the definition of ovulation in Drosophila mirror that of the mammalian field: the release of a mature oocyte out of the ovary, dependent upon temporally and spatially regulated follicle rupture.

To measure “ovulation” in Drosophila, it is important to remember a few key components of the ovulatory process. 1. For ovulation to occur, mature eggs must be present in the ovary. If there is a mutation that causes a reduction in oogenesis, there will be decreased ovulation. Waiting a few days post-eclosion and feeding females with wet yeast prior to an experiment should increase the amount of available eggs within the ovary. A characteristic phenotype of anovulation is egg retention – when there is a build-up of mature stage 14 egg chambers in the ovary. In this condition, females have many mature follicles but are unable to ovulate. Due to these phenomena, quantification of mature follicles present within the ovary is necessary for a read-out in egg laying experiments to attribute an “ovulation” defect. 2. Mating induces many behavioral and physiological changes, as described previously, therefore it must be considered before performing an ovulation assay. For example, mating increases octopamine through ovulin-induced mechanisms, and increases the rate of ovulation (Rubinstein and Wolfner, 2014). The status of mating for each female tested must be uniform throughout the experimental conditions. 3. Follicle rupture is necessary for ovulation to occur, however there are caveats to consider with only assaying this one aspect. Follicle rupture can be separated
from ovulation, for example if the oocyte ruptured within the ovary but wasn’t ejected out of the ovary (as described in chapters 2 and 4). 4. If females are expected to mate prior to an ovulation assay, it is important to confirm that they have normal copulation behavior. If a specific mutation impairs copulation behavior, it could also impact the post-mating response. If that is the case, then what would be measured wouldn’t be ovulation per se, rather would be a measurement of successful or unsuccessful copulation. 5. The transportation of an egg throughout the reproductive tract could influence the rate of ovulation, as demonstrated through experiments investigating oviduct secretions, contractions, and post-mating physiological and morphological changes. Egg transport is a complex process and therefore shouldn’t be a read-out for “ovulation”.

An egg is first ovulated and received in the lateral oviduct, and then it is transported to the uterus and eventually oviposited. These steps are separate from each other, and each can indicate its own set of phenotypes. If the oviduct lacks secretions or is deformed or uncoordinated in its contractions, the oocyte will be transported at a slower rate or not at all (Sun and Spradling, 2013). This situation provides an interesting example of the different metrics that could indicate different phenotypes. For example, if one is conducting an “egg location” assay, dissecting the reproductive tract to determine if there is an oocyte present, there could be a greater number of eggs within the reproductive tract. If the measurement of ovulation is “number of females with an egg in the reproductive tract”, there will be a high percentage of “ovulation”. Inherently, there will also be a reduced oviposition rate (decreased egg laying). If a female is laying fewer eggs, that could be indicative of a reduced “ovulation” rate. If one is interested in studying ovulation, it is essential that one remember the definition of ovulation before attributing a phenotype to an “ovulation” process. (Side note: CO2 is a common anesthetic to use, however we notice this somehow induces an ovulation event, and results in ~100% of control females to have a follicle within the reproductive tract. This is addressed in later chapters of this
dissertation. Many “control” experiments should be performed to eliminate the possibility of other confounding factors and determine the specific process of ovulation.)

As demonstrated within this introduction, there is a lack of knowledge regarding *Drosophila* ovulation proper, and there is a desperate need for a direct way to study ovulation. The general goal of this dissertation is to describe the role of the follicle cells in ovulation/follicle rupture in *Drosophila*, and the general hypothesis is the follicle cells have a dynamic, active role in ovulation in *Drosophila*. Hopefully, insight gained from this dissertation will provide the basis for using *Drosophila* as a model to study ovulation. However, before using it as a model to understand disease conditions, it is imperative to understand the normal biological processes. I will describe the stimulation for ovulation in *Drosophila* (Chapter 3, (Deady and Sun, 2015)), what the response is (Chapters 2 and 5, (Deady et al., 2015)), and how follicle cells gain competency to receive and respond to this stimulation (Chapter 4, (Deady et al., 2017)).
CHAPTER 2: MATRIX METALLOPROTEINASE IS REQUIRED FOR OVULATION AND CORPUS LUTEUM FORMATION IN DROSOPHILA


Author contributions: Conceived and designed the experiments: JS ACS. Performed the experiments: JS LDD WS SAM. Analyzed the data: JS LDD ACS. Wrote the paper: JS ACS.

INTRODUCTION

Ovulation, the liberation of a mature oocyte from the ovary, is one of the critical events of metazoan reproduction. In mammals, where ovulation has been studied most thoroughly, several important steps have been identified (Conti et al., 2012; Espey and Richards, 2006; Fan et al., 2012; Richards et al., 1998). First, among a cohort of mature ovarian follicles, a dominant follicle arises. Eventually, proteolytic enzymes are locally activated that digest a small part of the dominant follicle’s wall and extracellular matrix, releasing the oocyte into the oviduct (Ohnishi et al., 2005). Finally, residual follicular cells remodel the ruptured follicle into the yellowish corpus luteum, an endocrine body that secretes steroid hormone progesterone, estrogen, and other factors. While much has been learned, genetically testing the roles proposed for specific genes and pathways has been difficult. For example, the importance of matrix metalloproteinases (Mmps) in ovulation has not been demonstrated using knockout mice, possibly due to redundancy (Curry and Osteen, 2003; Curry and Smith, 2006; Gill et al., 2010; McCord et al., 2012). A genetically tractable system containing fewer redundant genes such as Drosophila would greatly facilitate ovulation studies. However, ovulation in Drosophila has not been well characterized and is not known to involve the same processes as mammalian ovulation.
The Drosophila female reproductive system is anatomically similar to mammals, having two ovaries connected by lateral and common oviducts to the uterus, where fertilization occurs and one egg is retained prior to laying (Fig. 1A, (Spradling, 1993)). Ovulation does not follow a simple cycle, however. Multiple eggs are laid when suitable food resources are available (Yang et al., 2008), and ovulation follows each oviposition to replenish the uterus. Egg laying and ovulation are extensively regulated by octopaminergic neural inputs (Lee et al., 2003; Monstirioti, 2003; Lee et al., 2009) and can be elicited by peptides transferred in semen from the male (Hasemeyer et al., 2009; Rubinstein and Wolfner, 2013; Yang et al., 2009; Yapici et al., 2008). Ovulation requires reproductive tract secretions controlled by the NR5a class nuclear hormone receptor Hr39 (Sun and Spradling, 2013). A mammalian ortholog, LRH-1, is required in mouse granulosa cells for ovulation, to maintain progesterone production in the corpus luteum and for decidual cell function in the uterus (Duggavathi et al., 2008; Zhang et al., 2013). These similarities highlight the potential value of Drosophila as a genetically tractable model of ovulation.
Figure 2.1 Drosophila follicle cells remain in the ovary following ovulation and form a corpus luteum.

(A) A schematic diagram of female reproductive system. The red squared area is the calyx where post-ovulatory follicle cells are located. (B) R47A04-Gal4 driving UAS-GFP expression (R47A04>GFP) is specifically in follicle cells of S14 egg chambers (mature follicles). (C) Follicle cells from post-ovulatory follicles (green; outlined) remain in the ovary after the egg enters the oviduct and form a corpus luteum (CL). (D) Hnt accumulates in S14 follicle cells after decreasing in S13. (E) Hnt expression continues in CL cells. (F and
F’) CL cells continue to express Arm, an adherens junction component (Red in F, white in F’) suggesting that they maintain their apical-basal polarity. (G) Ovaries of virgin females before egg laying. No pigmentation is found in the ovary. (H, I) Ovaries of mated females after egg laying. Yellow pigmentation (arrows) is found in the CL. A single CL is outlined. (J-L) Shade (Shd) is expressed in CL cells (dash-line outlined in J and J’) and S14 follicle cells (K, K’, and L). Shd expression co-localized with a mitochondria marker (yellow in J and K), but not with an endoplasmic reticulum marker (L).

Here, we show that similar follicle rupturing process occurs in Drosophila ovulation. Posterior follicle cells of a dominant mature egg chamber are first degraded and the residual follicle cells are squeezed toward the anterior while the oocyte moves posteriorly into the bilateral oviduct. These residual follicle cells remain in the ovary, accumulate yellowish pigmentation and persist for an extended period before degradation, which is reminiscent of the corpus luteum in mammals. We also demonstrated that Membrane-tethered Mmp2, but not Mmp1, functions in follicle cells to control follicle rupture. Our data suggest that the cellular and molecular regulation of ovulation has been more conserved than previously thought.

RESULTS

Follicles rupture to release the oocyte and form a corpus luteum

The fate of Drosophila follicle cells after ovulation has not been clearly described. If ovulation involves a programmed rupture of the follicular layer as in mammals, then most follicle cells would remain behind in the ovary after the egg is released into the oviduct. Alternatively, follicle cells might degenerate randomly or some might accompany the oocyte into the oviduct and the uterus. Using a mature (stage 14) follicle cell marker (Fig. 1B, Methods), we observed a cluster of GFP-labeled cells at the posterior end of each ovariole in the basal (calyx) region of ovary (Fig 1C). In contrast, few, if any, follicle cells leave the ovary upon ovulation because GFP-labeled cells were not seen associated with
eggs in the oviduct or uterus.

Post-ovulation mammalian follicles transform into the corpus luteum and similar behavior was reported previously in several other insects (Büning, 1994). Consequently, we termed the clusters of residual ovarian follicle cells “corpus luteum-like” (CL) cells and observed that they continue a program of gene expression. The zinc-finger transcription factor Hindsight (Hnt), a major follicle cell regulator (Sun and Deng, 2007), is upregulated in stage 14 follicle cells and is expressed in CL cells (Fig 1D and 1E). Expression of the adherens junction protein Arm (Fig 1F and 1F’), suggests that CL cells maintain apical-basal epithelial polarity. Most dramatically, CL cells acquire a yellowish pigmentation (Figure 1G-I) and express the ecdysone biosynthetic enzymes (Petryk et al., 2003; Warren et al., 2004) Shade in mitochondria (Figure 1J-L) and Phantom in the endoplasmic reticulum (ER; data not shown). All these indicate that Drosophila CL likely produces the steroid hormone ecdysone or 20-hydroxyecdysone, reminiscent of steroid hormone production by the mammalian corpus luteum. In addition, only one CL is present in each ovariole, hence it must either degrade in situ over time or be extruded from the ovariole when the next egg is ovulated.
Figure 2.2 Posterior follicle cells are removed to release the oocyte and initiate corpus luteum formation.

(A-D) Schematic (left panels) and real (right panels) expression patterns of Gal4 drivers in follicle cells of mature egg chambers and corpus luteum. Anterior follicle cells (B and D) reside at the anterior part of the corpus luteum, the middle follicle cells (C) reside at the middle portion of the corpus luteum, while the posterior follicle cells (D) are degraded in the corpus luteum. (E) An egg partially extruded into one lateral oviduct. (F-F’) DAPI stained calyx region showing the presence of two stage 14 trimming follicles (outlined) with no follicle cells at the posterior ends. The one with most posterior follicle cells trimmed was half-way in the lateral oviduct. The posterior end of follicle cells (brighter and larger nuclei pointed with an arrowhead) was marked by dashed lines, and the oviduct cells (faint and smaller nuclei) was marked by an arrow. F’ is the higher magnification of the squared region in F. (G) Histogram showing the number of follicles undergone trimming in mated (optimal ovulation) and unmated (no ovulation) females. Student’s T-test is used (*** P<0.001).
The organization of the CL reflects its origin in the follicle. All CL cells are labeled by a mature follicle cell driver, indicating that cells from other sources are absent (Figure 2A). Little cellular rearrangement occurs, since only anterior or middle cells of the corpus luteum were labeled by lines specifically expressed in anterior or middle stage 14 follicle cells (Figure 2B-C). Lines specifically expressed in the posterior follicle cells did not label the CL, suggesting that these cells were degraded during ovulation (Figure 2D).

**Trimming of posterior follicle cells and protrusion precedes ovulation**

Drosophila ovaries each usually contain at least 15 mature follicles, one per ovariole, oriented with their posterior ends facing the oviduct, raising the question of how one particular follicle is selected for ovulation. We examined ovary pairs from females cultured under conditions favorable for egg laying and found that at most one mature follicle protrudes significantly into a lateral oviduct (Figure 2E). The protruding follicle always lacked posterior follicle cells covering the part of egg inside the lateral oviduct (Figure 2F). We termed this process of losing posterior follicle cells as “trimming”. The trimmed follicle’s location indicates that trimming and protrusion represent preludes to ovulation. Frequently, another stage 14 follicle was present that had lost a smaller area of posterior follicle cells but did not protrude (Figure 2F-G and S1A-C), which likely represents the next follicle to ovulate. These observations show that a “dominant” follicle is selected in Drosophila well before ovulation, undergoes trimming, and awaits the next ovulation event while protruding into the lateral oviduct. In flies that were laying few eggs, for example in unmated females, up to 6 trimmed follicles could be present per female (Figure 2G), but the follicle with the greatest level of trimming continues to protrude into the oviduct and remains in a dominant position poised for ovulation.

**Gelatinase activity is associated with trimming and egg release**

The study of explanted mammalian follicles strongly implicates matrix
metallopeptinases (Mmp) as important contributors to oocyte release (Ohnishi et al., 2005). In these follicles, Mmp activity is localized to the apex region where rupture will later occur (Curry and Osteen, 2003). We carried out gelatinase assays in situ to measure localized Mmp activity within Drosophila follicles before and during ovulation. High localized activity was found at the posterior end of one mature follicle in each ovary pair while a second follicle sometimes had lesser activity (Figure 3A); the location of the activity correlated with the site of follicle cell trimming at the posterior (Figure 3B). As eggs begin to enter the oviduct, the fraction of the follicular surface with gelatinase activity (Figure 3C) increased from posterior to anterior and matched where follicle cells no longer reside (Figure 3C’). In eggs that have nearly separated from their follicle cells, gelatinase activity covered the entire surface (Figure 3D), however, the anterior and middle follicle cells remained in a mass at the base of the ovary (Figure 3D’). These data tightly associate Mmp activity with posterior follicle cell trimming and suggest that more anterior Mmp activity subsequently degrades just the extracellular matrix separating the oocyte from intact middle and anterior follicle cells.
Figure 2.3 Mmp2 is required for ovulation and CL formation.

(A) In situ zymography showing one preselected follicle with high gelatinase activity at its posterior end in the entire ovary pair. (B-D) Correlation of follicle cell trimming and gelatinase activity (green in B-D). During early (B), middle (C'), and late (D') ovulation,
Gelatinase activity is covering all the egg chamber area lost follicle cells. Posterior leading edge of the follicle cell layer was marked by dashed lines. Smaller nuclei (white in C' and D') are oviduct cells. (E-G) Egg laying (E), mature follicles in ovary (F), and the average ovulation time (G) of females of actGal4 or actGal4 driven Mmp1-RNAi, Mmp2-RNAi, or Timp expression in adult. (H-I) Follicle cell trimming in actGal4 control (H) and actGal4/Mmp2-RNAi (I) ovaries. Mature egg chambers with posterior follicle cell trimmed were outlined with solid line and the posterior edge of the residual follicle cells were marked with dashed line. Follicle cell nuclei are elucidated by DAPI signal. (J) A quantification of trimming follicles in ovaries. (K-L) More corpora lutea (arrowheads) are found in actGal4 control ovaries (K) than those in actGal4/Mmp2-RNAi ovaries (L) 6 hours after mating. One ovariole with three mature egg chambers was outlined in (K). (M) Number of eggs laid in 6 hours after mating. T-test is used (*** P<0.001, ** P<0.01, * P<0.05).

**Mmp2 is genetically required for ovulation and CL formation**

Drosophila has two genes encoding matrix metalloproteinases, *mmp1* and *mmp2*, and one Timp (*Tissue inhibitor of matrix metalloproteinase*)(Page-McCaw, 2008). Genetically reducing Mmp2 but not Mmp1 expression dramatically lowered egg laying (Figure 3E; Figure S2). Mature egg chambers accumulated in Mmp2 knockdown females (Figure 3F), indicating a block in ovulation, and the average time required to ovulate (see Methods) increased fivefold (Figure 3G and Table 1). Similarly, overexpressing Timp, a protein that inhibits both Mmp1 and Mmp2 activity, also decreased egg laying and increased egg retention and ovulation time (Figure 3E-G, Table 1). These data show that Mmp2 enzymatic activity is required for normal ovulation in Drosophila.

Mmp2 was also required for follicle trimming (Figure 3H, I and Table 2). The rate of trimming was reduced at least three-fold in Mmp2 knock down animals (Figures 3J and Table 2). In addition, Mmp2 knock down ovaries lacked corpus luteum structures (Figure 3K and 3L), and instead accumulated mature egg chambers (Figure 3L). Both Mmp2 knock down females and females expressing Timp, displayed severe egg laying defects even within 6 hours of mating (Figure 3M). Thus, Mmp2 activity is required in adult females for follicle cell trimming, ovulation, corpus luteum formation, and egg laying.
Mmp2 is expressed and functions in follicle cells to control ovulation

We generated an *in vivo* Mmp2::GFP fusion allele at its normal genomic location by swapping an in-frame GFP exon into a MiMIC transposon inserted within an Mmp2 coding intron (Figure S3). We also employed a Gal4 enhancer trap line (see Methods), which mimics Mmp2 expression during pupal imaginal disc eversion, to monitor Mmp2 transcription. Mmp2 fusion protein and RNA are specifically expressed in posterior follicle cells in all mature stage 14 follicles but not in earlier follicles (Figure 4A-C, S3C and S4A-D). Mmp2 is also expressed in some anterior follicle cells that help form dorsal eggshell structures. The reporters show expression at the posterior edge of surviving follicle cells during trimming, and in anterior and posterior corpus luteum cells (Figure 4D-E and S4D).

**Figure 2.4** Mmp2 functions in follicle cells to control ovulation.

(A-C) Mmp2 expression in mature egg chambers indicated by *Mmp2::GFP* fusion protein.
Mmp2 is highly expressed in both anterior (B) and posterior (C) follicle cells. Hnt (Red) is used to mark follicle cells. (D) Mmp2::GFP is expressed in anterior and posterior corpus luteum (outlined with dashed line). (E) Mmp2::GFP is expressed in the posterior edge of the trimming follicle cells when the egg is half way in the oviduct. The egg chamber is outlined by solid line, the posterior edge of follicle cells is marked by small dashed lines, and the corpus luteum is outlined by big dashed line. Smaller nuclei without Hnt expression are from oviduct epithelial cells. (F-G) The egg laying (F) and ovulation time (G) of females with 47A04-Gal4 or 47A04-Gal4 driven Mmp2-RNAi or Timp expression in mature follicle cells. T-test is used (**P<0.001, *P<0.05). (H-I) Ectopic Mmp2 in mature follicle cells is sufficient to cause egg release from ovary into abdominal cavity. (J) DAPI staining to indicate the follicle cell trimming of egg chambers released from ovary ectopic Mmp2 expression in (I). (K) Quantification of egg chamber trimming. Egg chambers already released from ovaries were collected from abdominal cavity of females with 47A04 driven Mmp1 or Mmp2 expression. 149 and 144 released egg chambers were collected from Mmp1 and Mmp2 flies respectively.

We interfered with Mmp2 expression specifically in mature follicle cells by using a mature follicle cell driver (R47A04) to express Mmp2 RNAi or to overexpress Timp and observed that ovulation and egg laying were defective (Figure 4F-G and Table 1). The defect is unlikely from disruption of Mmp2 in neurons as R47A04 is not expressed in sensory neurons innervating the female reproductive tract (Figure S4E-F). This is also supported by the observation that knocking down Mmp2 with a more restricted mature follicle cell driver (R42A05: expressed in posterior and anterior mature follicle cells; Figure 2D) showed a similar egg laying defect (Table 1), although one of lower severity. When Mmp2 was overexpressed in mature follicle cells, mature eggs rupture and are released into the female abdominal cavity (Figure 4H-I). Most such follicles lacked covering follicle cells (Figure 4J-K). When Mmp1 was ectopically expressed in mature follicle cells with the same Gal4 driver, fewer follicles ruptured into the abdominal cavity and most of those retained some follicle cells (Figure 4K). Consequently, Mmp2 is required in mature follicle cells to trim the follicular layer leading to ovulation, and its level of expression must be controlled or normal activity regulation may be overwhelmed.
**DISCUSSION**

Despite different biological strategies of ovulation in mice and Drosophila, our studies reveal strong similarities in the underlying mechanisms. In both species, a dominant follicle is selected, and its oocyte is released at an appropriate time by inducing Mmp proteolytic activity, either in the apex region (mouse) or at the follicle posterior (Drosophila). Mmp2 activation is likely controlled by pro-domain processing and may also be modulated at the level of protein secretion and/or by the presence of the endogenous inhibitor Timp. Pharmacological inhibition of Mmp activity prevents murine ovulation in vitro (Brännström et al., 1988; Reich et al., 1985), and in other vertebrate and primate follicles (Chaffin and VandeVoort, 2013; Ogiwara et al., 2005). However, knockouts of individual Mmp genes have not been reported to affect ovulation, presumably due to redundancy, although individual Mmp gene knockouts frequently have specific phenotypic effects (reviewed in (Gill et al., 2010)). In contrast, our studies clearly show a genetic requirement of Mmp2 but not Mmp1 for trimming, ovulation and CL formation.

The value of Drosophila for studies of ovulation is further illustrated by the discovery that after ovulation, residual follicle cells form a corpus luteum-like body. The corpus luteum (Latin for “yellow body”) was first described by Volcherus Coiter in 1573, but its relationship to ovulation rather than pregnancy was not understood until the early 19th century (Watt, 1915). The existence of a pigmented structure in insect ovaries was also recognized in the 19th century, at least in a few species (Büning, 1994). However, it has remained unclear whether a CL exists in Drosophila, whether it is a universal feature of insect oogenesis, whether the CL functions in reproduction, and whether any such functions have been conserved during evolution. Our studies indicate that a CL is formed in Drosophila and that Mmp2 activity is required for its production. The mammalian CL contains at least two cell types, small CL cells which are thought to be derived from thecal cells, and large CL cells that produce progesterone. Our gene expression studies suggest
that at least two cell types are also likely in the Drosophila CL (Tootle et al., 2011). Some anterior CL cells may derive from stretch cells, which never secrete eggshell proteins (Parks and Spradling, 1987).

The Drosophila CL may function at least in part by producing the steroid hormone, ecdysone or 20-hydroxyecdysone, indicated by continuous expression of P450 enzymes Phantom and Shade in CL cells. In addition, mated females have a higher ecdysone titer than unmated females (Harshman et al., 1999), consistent with the idea that the CL contributes substantially to ecdysone production. A common role in steroid hormone production might explain the conserved pigmentation of the CL. In mammals, carotenoid accumulation is beneficial to gametogenesis and is associated with steroid hormone production. These molecules may influence free radical balance, which might otherwise interfere with steroid production (Sawada and Carlson, 1996). Alternatively, carotenoids may simply accumulate due to the large amount of circulating lipoproteins that must be taken up to support steroid production (Schweigert, 2003). The easy of genetic manipulation in Drosophila may allow the biochemical nature and function of the yellow pigmentation in the CL-like bodies to be further characterized.

Finally, we propose that a major function of the Drosophila CL and possibly the mammalian CL, is to control the selection of subsequent dominant follicles, and hence to determine the order with which mature follicles are scheduled for ovulation. In their location at the posterior end of each ovariole, CL cells are well positioned to govern the orderly usage of mature follicles. If some aspects of CL cell secretory activity or responsiveness change with age, the corpus luteum in each ovariole would reflect the elapsed time since that particular ovariole was last used, allowing uniform usage of all stem cells to be ensured. Although the organization of follicles within the mammalian ovary is less obvious, signals from corpus lutea might influence the positioning and utilization of maturing follicles. Knowledge that fundamental aspects of ovulation are
similar in Drosophila and mammals will accelerate the study of these and many other questions.

**MATERIALS AND METHODS**

**Drosophila genetics**

Flies were reared on standard cornmeal-molasses food at 25°C unless otherwise indicated. The following Gal4 lines from the Janelia Farm collection (Jenett et al., 2012) were used to label follicle cells and corpus luteum cells: R47A04 (Oamb), R49E12 (5-HT2A), R10E05 (AstC-R2), and R42A05 (kay). To knockdown mmp1 or mmp2 or overexpress Timp in adult flies, actGal4/Cyo; tubGal80Δ5 virgin females were crossed to the following lines at 18°C and shifted to 29°C immediately after adult eclosion: UAS-mmp1RNAi (Bloomington Drosophila stock center, B31489), UAS-mmp1RNAi2 (Uhlirsova and Bohmann, 2006a), UAS-mmp1RNAi3 (Vienna Drosophila RNAi Center, V108894), UAS-mmp1E225A (a dominant negative form of Mmp1) (Glasheen et al., 2009), UAS-mmp2RNAi (Uhlirsova and Bohmann, 2006a), UAS-mmp2RNAi2 (VDRC, V107888), UAS-mmp2RNAi3 (BDSC, B31371), UAS-Timp (Page-McCaw et al., 2003). To knock down mmp1 or mmp2 or overexpress Timp in follicle cells of mature egg chambers, UAS-dcr2; R47A04 virgin females were crossed to the RNAi lines described above at 29°C. To overexpress mmp1 or mmp2 in mature follicle cells, R47A04 virgin females were crossed to UAS-mmp1 or UAS-mmp2 (Page-McCaw et al., 2003) at 21°C. Control flies were derived from specific Gal4 driver crossed to wild-type Oregon-R. Mmp2::GFP fusion genes were generated through recombinase mediated cassette exchange of MiMIC insertion (MI02914) in the third coding intron of mmp2 (Figure S3) (Venken et al., 2011). Mmp2::Gal4 line is from a Gal4 enhancer trap (Srivastava et al., 2007), and UAS-RedStinger (BDSC, B8547) and UASpGFP-act79B; UAS-mCD8-GFP were used as reporters.
B7194) and (Sun and Spradling, 2013)sqh-EYFP-ER (BDSC, B7195) were used for tracking mitochondria and endoplasmic reticulum, respectively.

Egg laying and ovulation time

Egg laying and ovulation was determined essentially as described. Virgin females were aged for four to five days and fed with wet yeast one day before experiments. To measure egg laying time (the average time between successive ovipositions), five females were mated to 10 Oregon-R males in each bottle covered with a molasses plate at 29 °C and five or more bottles were set up for each genotype. The molasses plates were replaced every 22 hours and the number of eggs laid was counted for 44 hours and used to determine the average time required per egg. Egg laying time (min) was then calculated as 22 hr x 60 min / eggs laid per 22hr. To determine ovulation time, about 30 single-pair matings with one virgin female and one Oregon-R male were carried out for each genotype at 29 °C for 6 hours, an interval sufficient for all females to reach a steady state level of ovulation and egg laying. Females were then frozen in -80 °C for four minutes, their reproductive tracts were dissected to identify eggs inside the reproductive tract, and the percentage of females with an egg in the uterus or actively ejecting out of the uterus (U%) was calculated. Free eggs were never observed in the common oviducts, indicating that eggs spend a negligible amount of time moving through the oviducts. Therefore, the egg laying time is partitioned into the uterus time (the average time eggs reside in the uterus or actively ejecting) and the ovulation time (the average time eggs prepare to be released from the ovary), which includes the time when the dominant follicle protrudes into the bilateral oviduct, because our study indicates that these eggs are in the process of ovulation and have not yet been released from the ovary (Figure 2E-F, 3D, and 4E). Uterus time was then calculated as egg laying time x U%, and ovulation time = egg laying time x (1 – U%). The 95% confidence intervals were calculated correspondingly.
**Follicle cell trimming**

Females were frozen in -80 °C for four minutes before dissection. Ovaries were dissected out immediately afterwards, fixed with 4% EM Grade paraformaldehyde, and stained with DAPI. Care was taken to make sure that two ovaries from single female were intact after staining and mounted in the same well on slides by carefully separating the ovarioles. The number of trimming follicles was scored according to the criteria that a quarter of the egg chamber at the posterior end has no follicle cells covering, and the number of mature follicles was scored according to their fully elongated dorsal appendage. The normalized trimming follicles were then calculated by the number of trimming follicles divided by the number of mature follicles in each female. For follicle cell trimming analysis in Figure 2G, three to four days old \( w^{1118} \) mated or unmated females were used. For analyzing trimming in females with Mmp knock down, three to four days old virgin females were mated with Oregon-R males for six hours before dissection. For trimming with misexpressing mmp1 and mmp2, follicles were directly collected from female abdominal cavity.

**Immunostaining, in situ zymography, and microscopy**

Antibody staining followed a standard procedure (Sun and Spradling, 2012). Briefly, tissues were fixed in 4% EM-Grade paraformaldehyde for 15 minutes and blocked in PBTG (PBS+ 0.2% Triton+ 0.5% BSA+ 2% normal goat serum). Primary antibodies were incubated overnight at 4 °C and secondary antibodies were incubated for two hours at room temperature, followed by DAPI staining. The following primary antibodies were used: mouse anti-Hnt (1: 75) and anti-Arm (1:40) from Developmental Study Hybridoma Bank, rabbit anti-Shd (1:250; a gift from Michael O’Connor) and anti-Phm (Warren et al., 2004) (1:250), rabbit anti-GFP (1:4000, Invitrogen), and rabbit anti-RFP (1:2000, MBL international). Secondary antibodies were Alexa 488 goat anti-rabbit and 546 goat anti-
mouse and anti-rabbit (1:1000, Invitrogen). Images were acquired using the Leica TCS SP5 confocal microscope, and assembled using Photoshop software (Adobe, Inc.). Images for yellow pigmentation of the corpus luteum were taken with the Macropod with Canon 6D camera and Olympus SZX16 stereomicroscope with Olympus DP72 color camera.

The *in situ* zymography technique for gelatinase activity was performed as previously described with minor modifications (Vidal et al., 2010). Ovaries were dissected in pre-warmed Grace’s media and incubated immediately in 100 μg/ml DQ-gelatin conjugated with fluorescein (Invitrogen) for an hour. To increase substrate penetration, the peritoneal muscle sheath was broken at the ovarian anterior. Ovaries were then fixed in 4% EM-Grade Paraformaldehyde for 15 minutes and mounted for visualization.

**ACKNOWLEDGEMENTS**

J.S. is supported by the University of Connecticut College of Liberal Arts and Sciences Start-up funds. A.C.S. is an Investigator of the Howard Hughes Medical Institute. We are grateful to Drs. Gerald Rubin, Andrea Page-McCaw, Dirk Bohmann, Tian Xu, and Michael O’Connor for sending us fly lines and antibodies, and to Drs. Chen-Ming Fan, Joseph LoTurco, Matt Sieber, and Robert Levis for comments and discussion on the manuscript.
TABLES AND SUPPLEMENTAL FIGURES FOR CHAPTER 2

Supplemental Figure 2-1 Expression and function of ecdysone biosynthetic enzymes in the corpus luteum and mature follicle cells.

(A-A") Phm is detected by antibody staining in mature follicle cells (stage 14) and corpus luteum cells, but weakly in stage-12 follicle cells. Phm expression is overlapped with Endoplasmic reticulum (ER) marker. (B) Knocking down genes encoding ecdysone biosynthetic enzymes (Shd, Phm, and Dib) with R47A04-Gal4 driver causes reduction of egg laying in two days. * P<0.05, ** P<0.01.

Supplemental Figure 2-2 Follicle cell trimming in paired ovaries.

(A) A table showing the number of mature or trimmed follicles in each ovary of the female flies rapidly laying eggs. (B-C) shows the two and only two trimmed follicles (outlined) from two ovaries of the same fly. The trimmed follicle in (B) protruded into the oviduct (arrow) and lost more follicle
cell covering at their posterior end than the one in (C).

Supplemental Figure 2-3 Two-day egg laying of females with reduced Mmp activity in adult.

Supplemental Figure 2-4 Generation and verification of Mmp2::GFP fusion protein

(A) Schematic diagram of Mmp2 genomic locus, MI02914 insertion site, and Mmp2::GFP
exchange cassette. (B) Recombinase-mediated cassette exchange of MI02914 and PCR verification of correct insertion of Mmp2::GFP fusion product. (C-D) Mmp2::GFP1 is the correct insertion and shows GFP expression in corpus luteum cells (outlined) and the posterior follicle cells of stage 14 egg chamber (arrow) but not stage 12 egg chamber. Mmp2::GFP2 is the incorrect insertion and does not show GFP expression.

Supplemental Figure 2-5 Expression of Mmp2-Gal4, 47A04-Gal4, and 42A05-Gal4

Gal4 expression was indicated by a UAS-mCD8-GFP reporter. (A-C) Mmp2-Gal4 is expressed in both anterior and posterior follicle cells of a mature follicle. (B) and (C) is the enlarged areas in
(A). (D) Mmp2-Gal4 is expressed in the anterior and posterior cells in corpus luteum (outlined). (E-F) 47A04-Gal4 is expressed in mature follicle cells (E) and the posterior end of the oviduct cells, but not in neurons innervating the reproductive tract (F). Oviduct is outlined in F. (G-H) 42A05-Gal4 is expressed in anterior (arrow) and posterior tip (arrowhead) follicle cells of mature egg chambers (G) and some interstitial cells in seminal receptacle (SR), but it is not expressed in the oviduct or uterus (Ut) or neurons innervating the reproductive tract. High auto-fluorescence was detected in the egg reside in the uterus. Oo: Oocyte.

Table 1. The effect of Mmp activity on egg laying, egg distribution in reproductive tract, and egg laying time.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Egg laying in 2 days*</th>
<th>Egg distribution in 6h</th>
<th>Egg laying time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>Eggs/ female/day</td>
<td>N</td>
</tr>
<tr>
<td>actGal4/+; tubGal80°/+</td>
<td>45</td>
<td>69.5 ± 0.5</td>
<td>30</td>
</tr>
<tr>
<td>actGal4/+; tubGal80°/+; UAS-mmp1RNi</td>
<td>25</td>
<td>62.9 ± 2.3*</td>
<td>27</td>
</tr>
<tr>
<td>actGal4/+; tubGal80°/+; UAS-mmp2RNi</td>
<td>40</td>
<td>20.0 ± 0.8***</td>
<td>30</td>
</tr>
<tr>
<td>actGal4/+; tubGal80°ts/UAS-Timp</td>
<td>25</td>
<td>40.1 ± 2.8***</td>
<td>28</td>
</tr>
<tr>
<td>UAS-dcr2/+; 47A04Gal4/+</td>
<td>50</td>
<td>64.1 ± 1.8</td>
<td>24</td>
</tr>
<tr>
<td>UAS-dcr2/+; 47A04Gal4/UAS-mmp2RNi</td>
<td>25</td>
<td>36.3 ± 3.8***</td>
<td>32</td>
</tr>
<tr>
<td>UAS-dcr2/+; 47A04Gal4/UAS-Timp</td>
<td>25</td>
<td>40.5 ± 1.5***</td>
<td>25</td>
</tr>
<tr>
<td>42A05Gal4/UAS-mmp2RNi</td>
<td>20</td>
<td>45.7 ± 3.5**</td>
<td></td>
</tr>
</tbody>
</table>

# one day =22h at 29 °C
* P<0.05, ** P<0.01, *** P<0.001
All data are mean ± 95% confidence interval. Ttest was used for egg laying, while Fisher's exact test was used for egg distribution.
Table 2. The effect of Mmp activity for follicle cell trimming.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>No. of females</th>
<th>6 hour post mating</th>
<th>Unmated virgin female</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of females</td>
<td>Mature eggs/female</td>
<td>Posterior trimmed eggs/female</td>
<td>Normalized trimming eggs/ female (%)</td>
</tr>
<tr>
<td>actGal4/++; tubGal80ts/+</td>
<td>14</td>
<td>21.6 ± 5.8</td>
<td>1.5 ± 0.9</td>
<td>7.2 ± 5.2</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>36.1 ± 9.8</td>
<td>1.8 ± 0.9</td>
<td>5.4 ± 3.2</td>
</tr>
<tr>
<td>actGal4/++; tubGal80ts/UAS-mmp1RNAi</td>
<td>24</td>
<td>31.3 ± 8.3</td>
<td>0.8 ± 0.9</td>
<td>2.5 ± 3.2</td>
</tr>
<tr>
<td>actGal4/++; tubGal80ts/UAS-Timp</td>
<td>20</td>
<td>34.7 ± 8.8</td>
<td>1.8 ± 1.6</td>
<td>5.3 ± 5.1</td>
</tr>
</tbody>
</table>

* P<0.05, **P<0.01, ***P<0.001
All data are mean ± SD. Ttest was used.
CHAPTER 3: A FOLLICLE RUPTURE ASSAY REVEALS AN ESSENTIAL ROLE FOR FOLLICULAR ADRENERGIC SIGNALING IN DROSOPHILA OVLATION

INTRODUCTION

Ovaries in organisms ranging from humans to insects are extensively innervated (Wojtkiewicz et al., 2014; Cisint et al., 2014; Middleton et al., 2006; Heifetz et al., 2014), and neuronal inputs likely play important roles in ovarian physiology (Gerendai et al., 2005). In mammals, ovaries are predominantly innervated by sympathetic fibers from the ovarian plexus nerve and the superior ovarian nerve (Aguado, 2002), which release norepinephrine (NE) locally and contribute to follicle development (Mayerhofer et al., 1997). Deregulation of sympathetic nerve outflow to ovaries is associated with polycystic ovary syndrome (PCOS), a common endocrine disorder leading to anovulatory infertility (Greiner et al., 2005; Lansdown and Rees, 2012). Despite the apparent importance of sympathetic innervation, however, it is not yet clear how the neuronal modulators/transmitters released from nerve termini affect ovulation (Weiner et al., 1975; Walles et al., 1977; Kobayashi et al., 1983; Kannisto et al., 1985; Schmidt et al., 1985; Wylie et al., 1985; Saller et al., 2012).

In Drosophila and other insects, the biogenic monoamines tyramine (TA) and octopamine (OA) act as functional counterparts to mammalian epinephrine and norepinephrine and regulate a variety of behaviors, including the fight-or-flight response, motivation, aggression, and reproduction (Maqueira et al., 2005; Roeder, 2005). Analogous to the adrenergic innervation in...
mammalian ovaries, *Drosophila* octopaminergic neurons innervate ovaries and the female reproductive tract (Fig 1A; (Middleton et al., 2006; Rodriguez-Valentin et al., 2006; Heifetz et al., 2014)). OA released from these neurons is essential for ovulation, as mutations that disrupt the enzymes required for OA synthesis, tyrosine decarboxylase 2 (Tdc2) and tyramine β-hydroxylase (TβH), completely block ovulation (Cole et al., 2005; Monastirioti, 2003; Monastirioti et al., 1996).

Four OA receptors have been identified in *Drosophila*: Oamb, Oct β1R, Oct β2R, and Oct β3R. Oamb is most closely related to mammalian α-adrenergic receptors, and the other three to β-adrenergic receptors (Han et al., 1998; Maqueira et al., 2005). Recent work demonstrated that *Oamb* and *Octβ2R* are important in egg laying and ovulation (Lee et al., 2003a, 2009; Lim et al., 2014). Oamb is widely expressed in the female reproductive system, including the ovary, with strongest expression observed in the oviduct (Lee et al., 2003). It is currently believed that OA activates receptors in the oviduct, inducing oviduct contraction and secretion, which ultimately regulates ovulation through an unknown mechanism (Lee et al., 2009; Lim et al., 2014; Rodriguez-Valentin et al., 2006). In addition to OA signaling, ovulation in *Drosophila* is affected by female reproductive gland secretions (Sun and Spradling, 2013) and by mating, which increases the ovulation rate by stimulating afferent nerve activity in the female reproductive tract (Hasemeyer et al., 2009; Heifetz et al., 2005, 2014; Lee et al., 2009; Rezával et al., 2014; Yang et al., 2008; Yapici et al., 2008). In particular, Ovulin transferred into the female reproductive tract after mating was recently shown to increase octopaminergic signaling and relax oviduct muscle (Rubinstein and Wolfner, 2013), consistent with the role of OA signaling in regulating muscle contraction. It is, however, not clear whether OA plays any direct roles in the ovary to control ovulation.
Figure 3.1 A novel ex vivo follicle rupture assay in Drosophila

(A) A schematic diagram representing the female reproductive system and ex vivo experiments. Mature follicle cells are marked by fluorescent proteins (red), and octopaminergic neurons are shown in green [3]. (B-C) Representative images show mature follicles after three-hour culture
Recent studies from our lab have shown significant conservation of the basic cellular and molecular mechanisms of ovulation from flies to mammals. Drosophila female contains two ovaries that are connected by the oviduct. Each ovary is organized into ovarioles, which have mature follicles (stage-14 egg chambers) at the posterior end toward the oviduct (Fig1A; (Spradling, 1993)). Each mature follicle has one layer of epithelial follicle cells surrounding the oocyte. During ovulation, posterior follicle cells are first trimmed to break the follicle-cell layer and to allow the oocyte to be released into the oviduct. The rest of the follicle cells remain at the end of the ovariole and form a corpus luteum (Deady et al., 2015). Similar to vertebrate ovulation (Curry and Osteen, 2003; Espey and Richards, 2006; Ogiwara et al., 2005), the entire follicle rupture requires matrix metalloproteinase 2 (Mmp2), a proteolytic enzyme expressed in posterior follicle cells of mature egg chambers but only activated during follicle rupture (Deady et al., 2015).

It is not yet clear what signals control Mmp2 activity, but it is clear that studying this question in Drosophila could yield important insights into the fundamental mechanism of ovulation.

Here, we developed the first ex vivo assay for follicle rupture in Drosophila and used it to investigate the role of octopaminergic signaling in this process. We found that OA directly activates its receptor Oamb on mature follicle cells to induce the breakdown of posterior follicle wall and ovulation. In addition, NE could partially substitute for OA, indicating an evolutionary
conserved role for follicular adrenergic signaling in ovulation. Finally, we demonstrated that follicular adrenergic signaling activates Mmp2 activity to control ovulation via the intracellular Ca$^{2+}$ as the second messenger. This is the first demonstration of a direct role of a neuromodulator in the control of follicle rupture during ovulation.

**RESULTS**

A novel ex vivo assay demonstrates the sufficiency of OA in inducing follicle rupture

Octopaminergic neurons innervate ovarioles extensively (Monastirioti, 2003), and OA receptor Oamb is transcribed in mature follicle cells according to *in situ* hybridization (Lee et al., 2003) microarray analysis (Fig S1; (Tootle et al., 2011)), and the expression of *R47A04-Gal4* (Pfeiffer et al., 2008), an *Oamb* enhancer element-regulated Gal4 driver, in mature follicle cells (Deady et al., 2105). We examined whether OA activates Oamb directly in mature follicle cells to induce follicle rupture. Mature follicles with an intact layer of follicle cells marked by *R47A04-Gal4* were isolated from ovaries (see methods) and cultured with OA or control media (Fig 1A). After three hours, follicles in control medium maintained an intact follicle-cell layer (Fig 1B). In contrast, about 80% of the follicles cultured with 5 μM of OA had shed their follicle-cell layer to the dorsal appendage at the anterior tip of the oocytes (Fig 1C); some were completely detached from the oocyte and floating in the medium. This phenomenon of shedding the follicle-cell layer, which we called follicle rupture in our ex vivo culture system, is reminiscent of what occurs during the ovulation process *in vivo* (Deady et al., 2105). The percentage of ruptured follicles with OA stimulation increased dramatically in the first two hours and reached a plateau at about three hours (Fig 1D). Extending the culture period neither increased the percent of ruptured follicles to 100% in the OA medium, nor allowed follicles in the control medium to reach the same level of rupture as OA-stimulated follicles (Fig 1D).
To validate that the follicle rupture in our ex vivo assay mimics the in vivo process, we video-recorded the entire rupturing process (Fig 1E and Movie S1). We found that posterior follicle cells were first trimmed, as we previously observed in vivo (Deady et al., 2105). The remaining follicle-cell layer was then squeezed toward the anterior dorsal appendage (Fig 1E and Movie S1). The entire rupturing process took 13.1 ± 5.0 minutes (Table S1), close to the estimated in vivo ovulation time of 11.2 ± 2.5 minutes (Table 1; (Deady et al., 2105)). Each mature follicle initiated the follicle rupture asynchronously, likely reflecting their asynchronous developmental stages; however, the kinetics of all ruptures was similar, with a very slow initial speed (Fig 1F). It took about 10 minutes to rupture through the posterior half of the oocyte, but only four minutes for the rest of the area (Fig 1E-F).

To further examine the quality of ex vivo ruptured oocytes, we determined whether these oocytes were activated. Mature oocytes released into the oviduct are activated and resistant to bleach treatment because their egg shells are hardened through cross-linking (Heifetz et al., 2001). This activation process can also be mimicked in vitro by culturing oocytes in hypotonic activation buffer (Mahowald et al., 1983; Page and Orr-Weaver, 1997). Using the established bleach assay (see methods), we found that oocytes from our ex vivo assay dissolved immediately after bleach treatment (n=96), indicating that they were not fully activated and their eggshells were not hardened. However, treatment with hypotonic activation buffer for 15 minutes can efficiently activate these ruptured oocytes (95%, n=150; Fig S2A-B), indicating these oocytes from our ex vivo system are of good quality and responsive to egg activation stimuli.

OA-induced follicle rupture is dose-dependent. Stimulation with 1 μM of OA had a minimal effect on follicle rupture, while stimulation with 20 μM of OA reached the maximal effect (Fig 1G), which led us to use 20 μM for all the following experiments. In contrast, stimulation with 20 μM of tyramine (TA), the immediate precursor of OA, had a much weaker effect on follicle rupture (Fig 1H), consistent with a previous report that OA, but not TA, is responsible for inducing ovulation.
(Monastirioti et al., 1996). Since NE is the counterpart of OA in mammals, we tested whether NE can also induce follicle rupture in our ex vivo assay. NE had only a minimal effect at lower doses (Fig 1I). Higher doses of NE could induce follicle rupture (Fig 1I), likely reflecting a differential binding properties of OA and NE to their respective receptors (Roeder, 2005). Nevertheless, these data suggest that OA and NE play a conserved role in regulating follicle rupture. In summary, we developed the first ex vivo assay to study follicle rupture in Drosophila, and our data suggest that OA is sufficient to induce follicle rupture in the absence of the oviduct and muscle function, as these tissues were excluded from our culture assay (68 mature follicles examined and none have ovariole muscle; Fig S3A-B).
Figure 3.2 Follicular Oamb is required for OA/NE-induced follicle rupture

(A-D) Representative images show mature follicles (marked by R44E10>GFP in follicle cells in red) after three-hour culture with 20 μM of OA (A-B) or NE (C-D). Mature follicles are from control (A and C) and Oamb mutant (B and D) females. (E) Quantification of Oamb mutant mature follicles in response to OA or NE stimulation. Four replicates were used for each genotype, except in Oamb−/− group with NE treatment, which has three replicates. (F-G) Quantification of follicle rupture after three-hour OA or NE treatment (20 μM). Mature follicles were derived from TβH (F) or Tdc2 (G) mutant females and marked by 47A04>RFP. All treatments have three replicates except for TβH+/− with NE treatment and Tdc2−/−, which have four replicates. (H-J) Oamb knockdown with R47A04-Gal4 blocks follicle rupture. Representative images show control (H) and Oamb-RNAi1 (I) mature follicles after three-hour culture with 20 μM of OA. Quantification of follicle rupture (J). The number of replicates for each condition in (J) is 3, 3, 3, 4, and 2. (K-M) Oamb knockdown with R44E10-Gal4 blocks follicle rupture induced by OA or NE. Representative
images show control (K) and Oamb-RNAi2 (L) mature follicles after a three-hour culture with 20 μM of OA. Quantification of follicle rupture (M). The number of replicates for each condition in (M) is 6, 5, 4, and 3. Student’s T-test was used (** P<0.01; * P<0.05).

Follicular Oamb is essential for OA/NE-induced follicle rupture

To identify the receptor responsible for OA/NE-induced follicle rupture, we focused on Oamb, which is essential for ovulation (Lee et al., 2003), and is the most highly expressed OA receptor in mature follicles (Fig S1). We verified the requirement of Oamb in ovulation with a new mutant allele (Oamb<sup>M12417</sup>), in which a MiMIC vector with splice acceptor(Nagarkar-Jaiswal et al., 2015) was inserted in the coding intron of Oamb gene to disrupt correct mRNA splicing (Fig S4). Females bearing this mutant allele laid significantly fewer eggs and took a much longer time to ovulate an egg (Table 1). We then isolated mature follicles from these females and applied OA stimulation ex vivo. Oamb mutant follicles showed severe defects in OA-induced follicle rupture compared to control follicles (Fig 2A-B, E). In addition, the Oamb mutation abolished the NE-induced follicle rupture (Fig 2C-E). The defective response of Oamb mutant follicles to OA/NE stimulation is not likely due to defective OA signaling in the oviduct or other organs, because follicles from TβH or Tdc2 mutant females are fully competent to OA/NE-induced follicle rupture (Fig 2F-G). These data indicate that Oamb in mature follicles is likely responsible for OA/NE-induced follicle rupture.

To test if Oamb functions directly in mature follicle cells, we knocked down Oamb specifically in these cells with RNA interference (RNAi) and then performed OA stimulation ex vivo. Oamb knockdown in mature follicle cells with R47A04-Gal4 severely disrupted OA-induced follicle rupture (Fig 2H-J). Since R47A04-Gal4 is regulated by an Oamb enhancer element (Pfeiffer et al., 2008), it could potentially be expressed in other Oamb-expressing cells, which may facilitate follicle maturation and ovulation. To exclude this possibility, we identified another Gal4
driver (R44E10-Gal4) expressed in mature follicle cells (Fig S5B-D). Compared to R47A04-Gal4, which is only expressed in late stage-14 follicles (Fig S5A), R44E10-Gal4 was expressed in all stage-14 follicles, slightly earlier than R47A04-Gal4. R44E10-Gal4 was not expressed in any tissues in the lower reproductive tract, nor in the neurons innervating the reproductive tract (Fig S5B, S5E, and S5F). Like mature follicles isolated using R47A04-Gal4, follicles isolated using R44E10-Gal4 were also responsive to OA/NE-induced follicle rupture (Fig S3E-F). In addition, mature follicles with R44E10-Gal4 driving OambRNAi showed similar unresponsiveness to OA or NE stimulation (Fig 2K-M). Taken together, these data suggest that follicular Oamb is required for OA/NE-induced follicle rupture ex vivo.
Figure 3.3 Follicular adrenergic signaling is required for ovulation and follicle cell trimming *in vivo*

(A-C) Egg laying (A), mature follicles in ovary (B), and the average ovulation and uterus time (C) is shown for control females or those expressing Oamb-RNAi in mature follicle cells driven by R44E10-Gal4. Student’s T-test was used (A-B; *** P<0.001; ** P<0.01; * P<0.05). (D-F) Follicle cell trimming is significantly reduced when follicular Oamb is knocked down by R44E10-Gal4 driving Oamb-RNAi1 expression (44E10>Oamb-RNAi1). Representative images show trimmed follicles in control (D) but not Oamb-RNAi (E) ovaries. Trimmed follicles are outlined with dashed yellow lines, and the posterior leading edge of the follicle-cell layer is marked by a straight red line. Quantification of trimmed follicles (F). (G-L) Follicle cell trimming is also significantly reduced in TβH (G-I) or Tdc2 (J-L) mutant females. See Tables 1 and 2 for the number of females analyzed and statistics.
Follicular adrenergic signaling is required for ovulation in vivo

To determine whether follicular adrenergic signaling is required for ovulation in vivo, we first analyzed the fecundity of females lacking follicular Oamb. Follicular Oamb-knockdown females with either R47A04-Gal4 or R44E10-Gal4 drivers laid significantly fewer eggs than control flies (Fig 3A and Table 1). The egg-laying defect is not caused by oogenesis problems, as mature follicles are abundant in these ovaries. In fact, Oamb-knockdown flies generally had more mature follicles in their ovaries (Fig 3B), indicating an ovulation defect. Indeed, Oamb-knockdown flies had a much longer ovulation time compared to control flies but did not show defects in transporting ovulated eggs into the uterus or ejecting them out of the uterus (Fig 3C and Table 1). These data strongly suggest that follicular Oamb is required for ovulation in vivo.

Trimming of posterior follicle cells is essential for ovulation and precedes follicle rupture (Deady et al., 2105). We investigated the role of follicular adrenergic signaling in this trimming process. Posterior trimmed follicles were readily observed in the ovaries of control females six hours after mating, and they account for 9% of the total mature follicles in each female (Fig 3D, 3F and Table 2), consistent with our previous analysis (Deady et al., 2105). In contrast, the percentage of posterior trimmed follicles was reduced three fold in females lacking follicular Oamb (Fig 3E-F and Table 2), indicating its essential role in follicle trimming. This is consistent with our observation that posterior follicle cells remain intact in Oamb-knockdown follicles even after three hours of OA stimulation ex vivo (Fig 2I and 2L). Furthermore, the percentage of trimmed follicles also decreased in flies that lacked the ability to produce OA; we saw a reduction to 2.4% and 0.4% in TβH and Tdc2 mutant females, respectively (Fig 3G-L and Table 2). This reduction of trimmed follicles was not only observed in mated females, but also in virgin females (Table 2). Taken together, these data suggest that follicular adrenergic signaling is required for posterior follicle cell trimming.
Figure 3.4 Adrenergic signaling activates Mmp2 to regulate ovulation

(A-C) In situ zymography shows increased Mmp activity in mature follicles after three-hour culture with 20 μM of OA. Mmp activity is indicated by Gelatin-fluorescein (green in A and B). The percentage of follicles with posterior Mmp activity is quantified in (C; *** P < 0.001). Three and four replicates were used for OA- and OA+ groups, respectively. (D-F) Expression of Mmp2-RNAi or Timp driven by R44E10-Gal4 prevents follicle rupture in response to OA or NE (*** P <0.001 and ** P < 0.01). The number of replicates used for each condition is 6, 5, 6, 4, 3, and 3. (G-I) Expression of Mmp2-RNAi or Timp driven by R47A04-Gal4 prevents follicle rupture in response to OA or NE. All experiments were performed in four replicates except Mmp2-RNAi, which have
three replicates. (J-L) Ovaries are shown for the Oamb mutant (J), the Oamb mutant with ectopic expression of Mmp2 driven by R44E10-Gal4 (K), and the Oamb heterozygous with ectopic Mmp2 expression (L). Mature eggs were released into the female abdominal cavity.

**Adrenergic signaling activates Mmp2 to regulate ovulation**

The crucial role of Mmp2 in trimming of posterior follicle cells (Deady et al., 2105) prompted us to investigate the relationship between follicular adrenergic signaling and Mmp2 activity. It is unlikely that adrenergic signaling regulates Mmp2 expression, as Mmp2 was readily detected in the posterior follicle cells of TβH mutants (Fig S6A-B). To test whether OA regulates Mmp2 activity, we examined gelatinase enzymatic activity in the OA-induced ex vivo ovulation assay using in situ zymography (Curry & Osteen, 2003; Deady et al., 2015). About 20% of mature follicles cultured in a control medium had gelatinase activity at their posterior end (Fig 4A, 4C, S6C, S6G). In contrast, more than 70% of mature follicles stimulated with OA had gelatinase activity (Fig 4B-C, S6D, S6G). The entire eggshells of ruptured oocytes were coated with Mmp-activated gelatin-fluorescein (Fig 4B, S6D), as we observed in vivo (Deady et al., 2105). In addition, OA-induced gelatinase activity was blocked in mature follicles with Oamb knockdown or misexpression of Timp, an endogenous inhibitor of Mmp2 (Page-McCaw et al., 2003), in follicle cells (Fig S6E-G). These data indicate that OA-Oamb signaling is sufficient to induce Mmp2 activation.

To determine whether Mmp2 activity is required for OA-induced follicle rupture, we isolated mature follicles containing follicle cell-specific Mmp2 knockdown and cultured them in the OA medium. These follicles did not respond to OA stimulation, and their posterior follicle cells remained intact (Fig 4D-I). In addition, Mmp2 knockdown in follicle cells also abolished the NE-induced follicle rupture (Fig 4F and 4I). Furthermore, misexpression of Timp in mature follicle cells completely prevented follicle rupture ex vivo (Fig 4F and 4I). Therefore, Mmp2 activity in
mature follicle cells is essential for OA/NE-induced follicle rupture *ex vivo*, consistent with its essential role in follicle trimming and ovulation *in vivo* (Deady et al., 2015).

To confirm that Mmp2 acts downstream of adrenergic signaling in follicle trimming and rupture, we attempted to rescue the defect of follicle rupture in *Oamb* mutant flies with ectopic expression of Mmp2 in mature follicle cells. *Oamb* mutant females had two intact ovaries, which contain a large number of mature follicles (Fig 4J). In contrast, follicular misexpression of Mmp2 in *Oamb* mutant females caused the breakdown of the ovariole muscle sheath and the release of mature follicles into the abdominal cavity (Fig 4K). Further examination of these released follicles demonstrated that 99% of them (n=70) had no follicle-cell covering, similar to follicles released upon misexpression of Mmp2 in *Oamb* heterozygous or wild-type females (Fig 4L; (Deady et al., 2015)). Therefore, Mmp2 is sufficient to induce follicle rupture in the absence of adrenergic signaling. Together, our data indicate that follicular adrenergic signaling activates Mmp2 to control follicle trimming and ovulation.

**Intracellular Ca\(^{2+}\) acts as the second messenger downstream of follicular adrenergic signaling to induce follicle rupture**

OA-Oamb interaction can induce transient increase of intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) (Han et al., 1998). To determine whether OA evokes Ca\(^{2+}\) release in mature follicle cells to induce follicle rupture, we first monitored the [Ca\(^{2+}\)]\(_i\) using a genetically encoded calcium sensor (see method). Fluorescent intensity of the calcium sensor expressed in mature follicle cells rose significantly around six minutes after OA administration in our *ex vivo* culture system (Fig S7 and Movie S2). To determine whether Ca\(^{2+}\) is required for OA-induced follicle rupture, we pretreated mature follicles with BAPTA-AM, an intracellular Ca\(^{2+}\) chelator, before OA stimulation. Two hundred μM BAPTA-AM treatment significantly perturbed the OA-induced follicle rupture (Fig 5A-C). To determine whether Ca\(^{2+}\) is sufficient to induce follicle rupture, we stimulated mature follicles with ionomycin, a potent ionophore for increasing [Ca\(^{2+}\)]. Ionomycin is potent to induce follicle
rupture even at 5 μM concentration (Fig 5D-F), lower than the dose typically used in the field (Wong et al., 2005). Taken together these data suggest that the increase of \([\text{Ca}^{2+}]_i\) is both necessary and sufficient to induce follicle rupture.

To further test whether \(\text{Ca}^{2+}\) is the second messenger of follicular adrenergic signaling for Mmp2 activation and follicle rupture, we set to examine whether ionomycin is sufficient to induce rupture of follicles lacking follicular Mmp2 or Oamb, which do not respond to OA stimulation. Ionomycin only partially induces follicle rupture when Mmp2 is knocked down in mature follicle cells and is not able to induce any rupture when Timp is overexpressed (Fig 5G-I). In contrast, ionomycin is able to induce follicle rupture in both control and Oamb mutant follicles at the equal level (Fig 5J-L). Together, our data indicate that follicular adrenergic signaling activates Mmp2 to control follicle trimming and ovulation likely via intracellular \(\text{Ca}^{2+}\) (Fig 5M).
Figure 5

(A-C) Pretreatment of BAPTA-AM blocks OA-induced follicle rupture. Representative images show mature follicles treated with DMSO (A) or BAPTA-AM (B) followed a three-hour stimulation with 20 μM of OA. Ruptured follicles were quantified in C. Three replicates are used for each condition. (D-F) Ionomycin is sufficient to induced follicle rupture. Representative images show follicles after three-hour culture with ethanol (D) or 5 μM of ionomycin. Ruptured follicles after different doses of ionomycin treatment are quantified in F. All conditions have three replicates except in 5 μM, which has four replicates. (G-H) Representative images of Mmp2-RNAi (G) and UAS-Timp (H) follicles treated with 5 μM of ionomycin for three hours. (I) Quantification of ruptured follicles with Mmp2 knockdown or Timp overexpression in mature follicle cells in response to 20
μM of OA or 5 μM of ionomycin stimulation. All conditions have three replicates except for Timp overexpression with ionomycin treatment, which has six replicates. (J-L) Ionomycin, but not OA, is sufficient to induce rupture in Oamb mutant follicles. Representative images show Oamb+/− (J) and Oamb−/− (K) follicles after three-hour culture with ionomycin. (L) Quantification of ruptured follicles after three-hour culture with 20 μM of OA or 5 μM of ionomycin. The number of replicates for each condition is 4, 4, 3, and 5. (M) A cartoon showing the model of follicular adrenergic signaling in Mmp activity and follicle rupture. Octopaminergic neurons are shown in green.

**DISCUSSION**

**The first ex vivo follicle rupture assay in Drosophila**

Ovulation, an essential step in metazoan reproduction, has been extensively studied in mammals over the past several decades (Conti et al., 2012; Fan et al., 2009, 2012). However, progress in the field has been hindered by the limited ability of mammalian model systems to be genetically manipulated. Thus it is still unclear how follicles break their wall in a highly regulated spatio-temporal manner to allow release of oocytes. The model organism *Drosophila* offers a wealth of tools for genetic manipulation, but to date few specific readouts for *Drosophila* ovulation has been developed. Previous studies of *Drosophila* ovulation have used readouts such as egg laying, percentage of females with eggs in the reproductive tract, or egg retention (Heifetz et al., 2005; Lee et al., 2003a; Lim et al., 2014; Monastirioti, 2003). These readouts have been very useful to follow the egg-laying process and to determine on which stage in the process a specific effector works. However, it is not reliable to predict ovulation defects just based on one individual parameter, because they can be affected not only by defects in ovulation but also by changes in other biological processes in the reproductive system such as oogenesis, oviposition, and egg transportation (Bloch Qazi et al., 2003). As such, we recently combined these parameters to estimate ovulation time (Deady et al., 2015; Sun & Spradling, 2013). In the present study, we developed a novel *ex vivo* follicle rupture assay in *Drosophila* and demonstrated that OA-induced follicle rupture in this assay is similar to the rupturing process *in vivo*. This assay gave us the unprecedented ability to visualize the entire process of follicle rupture and quantify its kinetics.
Further genetic evidence illustrated that genes required for ex vivo follicle rupture are also involved in vivo, including Oamb and Mmp2. Our ex vivo assay represents the most simple, specific, and reliable method for measuring rupturing ability of mature follicles. In conjunction with the powerful genetic tools available in Drosophila, this ex vivo assay will allow genetic screens to identify candidate genes involved in follicle rupture, thus opening new avenues for ovulation research.

A direct role for octopamine signaling in Drosophila ovulation

Octopamine, a biogenic amine derived from tyrosine, has been identified as essential for ovulation in Drosophila (Monastirioti et al., 1996). The major source of OA is octopaminergic neurons innervating the female reproductive system, and previous studies showed that restoring TβH specifically in these neurons rescues the ovulation defect caused by TβH mutation (Monastirioti, 2003). Due to its effects on muscle contraction, OA was proposed to regulate ovulation by inducing the contraction of ovarian muscle and relaxation of oviduct muscle (Lee et al., 2009; Lim et al., 2014; Middleton et al., 2006; Rodriguez-Valentin et al., 2006; Rubinstein and Wolfner, 2013).

Ovarian smooth muscle contraction was also proposed to regulate ovulation in mammals in the early 1980’s (Martin and Talbot, 1981; Walles et al., 1975, 1977). However, subsequent work suggest that ovulation requires the active proteolytic degradation of the follicle wall rather than passive muscle contraction (Tsafriri, 1995; Espey and Richards, 2006; Brown et al., 2010). At least three families of proteolytic enzymes are involved in this process, including matrix metalloproteinases (Curry and Smith, 2006; Ohnishi et al., 2005). Pharmacological blockage of any of these enzymes results in inhibition of follicle rupture.

Our recent work suggested that Drosophila also requires proteolysis for breaking the follicle wall and ovulation (Deady et al., 2015), and in this way shares similarities with mammalian ovulation at both the cellular and molecular level (Deady et al., 2015; Sun & Spradling, 2013).
These new insights into *Drosophila* ovulation process lead to the speculation that octopaminergic signaling may play a direct role on the follicle in controlling ovulation in addition to its role on muscle contraction. Here, we demonstrate that OA-Oamb signaling in mature follicle cells directly regulates follicle wall degradation, follicle rupture, and ovulation by activating key enzyme Mmp2. Furthermore, our pharmacological data suggest that OA-Oamb signaling likely fulfill these functions via intracellular Ca$^{2+}$ as the second messenger. It is intriguing that [Ca$^{2+}$], also rises after NE and gonadotropin stimulation in human granulosa cells (Föhr et al., 1993) and that perfusion of a Ca$^{2+}$ chelator in rabbits significantly reduces gonadotropin-induced ovulatory efficiency (Kitai et al., 1985). Given adrenergic innervation of ovaries observed throughout metazoans, it is plausible to speculate that follicular adrenergic signaling plays conserved roles in regulating Mmp activity and ovulation (See below).

**Conservation of ovarian adrenergic signaling in ovulation**

Adrenergic innervation of the ovary has long been found in mammals including humans. The role of adrenergic signaling in ovulation has been studied as early as the 1970’s. The neurotransmitter norepinephrine (NE) reaches the highest level in peripheral plasma during ovulation (Blum et al., 2004) and is enriched in the follicular fluid of preovulatory follicles compared to in peripheral plasma in healthy women (Bòdis et al., 1992; Itoh et al., 2000; Saller et al., 2012). Functional adrenergic receptors are expressed in mammalian ovarian follicular cells (Föhr et al., 1993; Itoh and Ishizuka, 2005; Kannisto et al., 1985). Ovarian perfusion of adrenergic agonists or antagonists influences the ovulation rate in rabbits and rats (Kobayashi et al., 1983; Schmidt et al., 1985). It has been speculated that adrenergic signaling regulates ovulation by stimulating muscle contraction or by increasing production of reactive oxygen species (Saller et al., 2012; Walles et al., 1975). In contrast to this view, ovarian sympathetic denervation does not affect ovulation in rabbits and rats (Weiner et al., 1975; Wylie et al., 1985); instead, it rescues ovulation defect in a rat model of PCOS (Barria et al., 1993; Morales-Ledesma et al., 2010), which is
associated with increased sympathetic inputs to the ovary (Greiner et al., 2005; Lansdown and Rees, 2012). It is not clear why a discrepancy exists between the effects of surgical denervation and of pharmacological agents. Thus, no consensus has been reached in regard to the role of ovarian adrenergic signaling in mammalian ovulation.

Instead of regulating ovarian smooth muscle contraction, the results of the present study suggest an alternative pathway for ovarian NE to regulate ovulation. NE likely activates adrenergic receptors in granulosa and theca cells (equivalent to Drosophila follicle cells) in mammalian periovulatory follicles, which activates Mmp enzymatic activity at the apex (Curry and Osteen, 2003) where mature oocytes rupture through. A surgical denervation may cause tissue damage and activate Mmps directly, bypassing the requirement of follicular adrenergic signaling. Future studies, using both mammalian and Drosophila genetic tools, will identify fundamental mechanisms of adrenergic signaling in ovulation.

MATERIALS AND METHODS

Drosophila genetics

Flies were reared on standard cornmeal-molasses food at 25°C unless otherwise indicated. Oamb^{M12417} is a MiMIC line inserted in the coding introns of all Oamb splicing isoforms (Nagarkar-Jaiswal et al., 2015), and Oamb^{M12417/Df(3R) BSC141} was used to characterize the Oamb mutant phenotype. TbH^{M18} (Monastirioti et al., 1996) and Tdc2^{ROS4} (Cole et al., 2005) were kindly provided by Dr. Mariana Wolfner. All RNAi-knockdown experiments were performed at 29°C with UAS-dcr2 to increase the efficiency of RNAi. R47A04-Gal4 (Oamb) and R44E10-Gal4 (lli) from the Janelia Gal4 collection (Pfeiffer et al., 2008) were used for misexpressing genes or RNAi in mature follicle cells. The following RNAi or overexpressing lines were used: UAS-Oamb^{RNAi1} (V2861) and UAS-Oamb^{RNAi2} (V106511) from the Vienna Drosophila Resource Center; UAS-Oamb^{RNAi3} (B31233) and UAS-Oamb^{RNAi4} (B31171) from the Bloomington Drosophila Stock
Center; UAS-Mmp2RNAi (Uhlirsova and Bohmann, 2006b); UAS-Mmp2 (Page-McCaw et al., 2003); and UAS-GCaMP5G (Akerboom et al., 2012). UASpGFP-act79B; UAS-mCD8-GFP (Deady et al., 2105) was used to analyze Gal4 expression in both germline and somatic cells, as well as neurons. UAS-GFPnls and UAS-RFP were used for follicle isolation. Control flies were derived from specific Gal4 drivers crossed to Oregon-R or yv; attP2 (B36303). The Mmp2::GFP fusion allele in the Mmp2 endogenous locus was used for detecting Mmp2 protein expression (Deady et al., 2015).

**Ex vivo follicle rupture, Ca^{2+} imaging, in situ zymography, and egg activation assays**

For the *ex vivo* follicle rupture assay, 4-6-day-old virgin females were used to isolate mature follicles, and follicle cells were fluorescently labeled using *R47A04-Gal4* or *R44E10-Gal4*. Ovaries were dissected in Grace’s medium and ovarioles were separated from each other using forceps. This process will break the ovariole muscle sheath and release mature follicles. Mature follicles with an intact follicle-cell layer and completely dissociated from younger follicles were immediately transferred to new Grace’s medium to minimize their exposure to endogenous biogenic amines during dissection. With this method, we can isolate about 10 mature follicles/female and isolated mature follicles are no longer associated with ovariole or oviduct muscle sheaths (Fig S3). Within one hour, isolated mature follicles were subsequently cultured in culture media (Grace’s medium, 10% fetal bovine serum, and 1X penicillin/streptomycin) supplemented with the indicated concentration of OA, TA, NE (Sigma), or ionomycin (dissolved in ethanol; Cayman Chemical). For chelating intracellular Ca^{2+}, isolated mature follicles were treated with BAPTA-AM (dissolved in DMSO; Cayman Chemical) for 30 minutes before OA culture. All cultures were performed at 29°C, the same condition as flies were maintained, to enhance Gal4/UAS expression. About 25-30 follicles were used for each culture group and the percentage of ruptured follicles was then calculated as one data point. Typically, three-six replicates were used for each genotype or treatment; data were represented as mean percentage
± standard deviation (SD); and Student’s T-test was used for statistical analysis. Ruptured follicles were defined as those losing more than 80% follicle-cell covering. With the exception of Figure 1D, all data were collected at the end of the three-hour culture.

For Ca²⁺ imaging and follicle rupture kinetics, Video images were captured at 0.2 frame/second (FPS) with a sCOMS camera (PCO.Edge) installed in a Leica MZ10F fluorescent stereoscope. To examine the kinetics of follicle rupture, mature follicles were cultured in 20 μM of OA medium for 20 minutes at 29°C before video recording, which was performed at room temperature. Each ruptured follicle was analyzed frame-by-frame manually to determine the ruptured distance between the posterior tip of the oocyte and the posterior leading edge of the follicle-cell layer using ImageJ. The percent of ruptured distance was then calculated as the ruptured distance divided by the length of the oocyte from the anterior to posterior tip. Because of the asynchronous onset of follicle rupture, data were normalized at the time point when follicles reach 50% ruptured area.

In situ zymography for detecting gelatinase activity was performed as previously reported with minor modifications (Deady et al., 2105). 50 μg/ml of DQ-gelatin conjugated with fluorescein (Invitrogen) was added into the culture media with or without OA for three hours. After a quick rinse, mature follicles with posterior fluorescent signal were directly counted. For egg activation, ruptured oocytes were treated with hypotonic activation buffer (Page and Orr-Weaver, 1997) for 15 minutes and treated with 50% bleach for three minutes. Unbroken oocytes were then counted.

Egg laying, ovulation time, and follicle cell trimming

Egg laying, ovulation time, and follicle cell trimming were performed as previously described (Deady et al., 2015; Sun & Spradling, 2013). In brief, 4-6-day-old virgin females fed with wet yeast for one day were used. For egg laying, five females were housed with ten Oregon-R males in one bottle to lay eggs on grape juice-agar plates for two days at 29°C. After egg laying, ovaries were dissected and mature follicles in these ovaries were counted. The number of eggs
on the plates was then counted, which was used to calculate the average time for laying an egg (egg-laying time). The egg-laying time was partitioned into the ovulation time and the uterus time (the time egg spent in the uterus and during oviposition). The partition ratio was determined based on the percentage of females having eggs in the uterus at six hours after mating. To do so, ten virgins were placed in a vial with 15 Oregon R males for six hours at 29°C, frozen for 4.5 minutes at -80°C, and then dissected to examine the reproductive tract. For follicle cell trimming, virgin or mated females were frozen for 4.5 minutes at -80°C, and ovary pairs were dissected, fixed, stained with DAPI, and mounted carefully to preserve the posterior end of mature follicles. Trimmed follicles were defined as more than a quarter of oocytes at the posterior end lacking follicle cell covering. Normalized trimming follicles were then calculated by the number of trimming follicles divided by the number of mature follicles in each female.

**Immunostaining and microscopy**

Immunostaining was performed following a standard procedure (Sun and Spradling, 2012), including fixation in 4% EM-grade paraformaldehyde for 15 minutes, blocking in PBTG (PBS+ 0.2% Triton+ 0.5% BSA+ 2% normal goat serum), and primary and secondary antibody staining. Mouse anti-Hnt (1:75; Developmental Study Hybridoma Bank) and rabbit anti-GFP (1:4000; Invitrogen) were used as primary antibodies, and Alexa 488 goat anti-rabbit and 546 goat anti-mouse (1:1000, Invitrogen) were used as secondary antibodies. Images were acquired using a Leica TCS SP8 confocal microscope or Leica MZ10F fluorescent stereoscope with a sCOMS camera (PCO.Edge), and assembled using Photoshop software (Adobe, Inc.).

**ACKNOWLEDGEMENTS**

We thank Drs. Mariana Wolfner, Hugo Bellen, Gerald Rubin, Dirk Bohmann, Andrea Page-McCaw, and Allan Spradling for sharing fly lines, the Bloomington *Drosophila* Stock center and the Vienna *Drosophila* Resource Center for fly stocks, and the Developmental Study Hybridoma Bank for antibodies. We are grateful to Drs. Laurinda Jaffe, Joseph LoTurco, Akiko Nishiyama,
Anastasios Tzingounis, and Robert Gallo for insightful discussions about this project and comments on the manuscript, and Dr. Tzu-Ting Chiou, Wei Shen, and Elizabeth Knapp for technical support. J.S. is supported by the University of Connecticut Start-up and Scholarship Facilitation funds.
Supplemental Figure 3-1 Expression of OA/TA receptors in stage 10, 12, and 14 follicles

Data were mined from previous microarray analysis. Two independent datasets of stage 10 and 14 follicles were used for calculating mean expression and standard deviation.

Supplemental Figure 3-2 Ruptured oocytes can be activated by hypotonic buffer.

(A-B) Hypotonic buffer-treated ruptured follicles before (A) and after (B) bleach treatment. Eggs tolerant to bleach treatment were activated.
Supplemental Figure 3-3 Isolated mature follicles do not contain ovariole muscle sheath.

(A) Intact ovaries stained with phalloidin (green in A and white in A') show ovariole muscle sheath wrapping around the ovarioles. (B) Isolated mature follicles stained with phalloidin (green in B and white in B') are not surrounded by the ovariole muscle sheath.

Supplemental Figure 3-4 Molecular characterization of OambMI12417 allele

The upper panel shows the genomic organization and alternative splicing of Oamb gene. The MiMIC insertion in OambMI12417 allele is also indicated. Orange boxes depict the coding exons and red arrows indicate PCR primer. The bottom panel shows RT-PCR results using isolated mature follicles. OambK3, but not OambAS, is expressed in OambMI12417/+ mature follicles.
(Lane 3 and 5), consistent with previous report. In contrast, neither of these isoforms are expressed in *Oamb*M12417/DF(3R) *BSC141* mature follicles (Lane 4 and 6). The primers used were: CCGCTTCAAGGGACAGTATC (rp49-F), GACAATCTCCTTGCCTTTCT (rp49-R), TGACCAACGATCGGGGTAT (K3-F), ATGCACATATGAGCTGGGA (K3-R), AGAAGCAGGAGAGCCATCAA (AS-F), TTGATCTTGTCTGGTGGTG (AS-R).

Supplemental Figure 3-5 Expression of R47A04-Gal4 and R44E10-Gal4 and R44E10-Gal4-labeled follicles in response to OA and NE.

(A) R47A04-Gal4 driving UAS-GFP expression (47A04>GFP) in follicle cells of late, but not early, stage-14 egg chambers. Early stage-14 egg chambers are recognized based on remnant of nurse-cell nuclei (arrows). (B-F) R44E10-Gal4 expression (44E10>GFP) in the female reproductive system. R44E10-Gal4 is expressed in follicle cells of all stage-14 egg chambers (B and D), but not in younger egg chambers (B and C). It is not expressed in any region of the oviduct (B, E and F), nor in the uterus, spermathecae, or neurons innervating the reproductive tract (B.
and F). The oviduct is outlined by a white line in E and F and an asterisk in B. The oocyte halfway in the oviduct is outlined by a dashed yellow line, and the posterior leading edge of the follicle-cell layer is marked by a red line in E. An arrow points to the spermathecae in F. Hnt (red) is a zinc-finger transcription factor expressed in mature follicle cells [37] and spermathecal glands [28]. (G-H) The dose response of R44E10-Gal4-labeled mature follicles to OA (G) and NE (H) in follicle rupture. The reduced response with R44E10-Gal4 labeling than R47A04-Gal4 is likely because it enables the isolation of slightly early stage-14 egg chambers. All conditions have three replicates except 0 and 20 μM OA, which have five replicates.

Supplemental Figure 3-6 Follicular adrenergic signaling activates Mmp2 enzymatic activity but not Mmp2 expression.

(A-B) Mmp2::GFP is expressed normally in posterior follicle cells of control (A) and TβH mutant (B) follicles. (C-F) Gelatinase activity in mature follicles after three-hour cultures without (C) or with (D-F) 20 μM of OA. Mature follicles were from control females (C-D) and females with 47A04-Gal4 driving Oamb-RNAi1 (E) and Timp (F) expression. (G) Quantification of gelatinase activity
from (C-F). ** P<0.01. All conditions have three replicates.

Supplemental Figure 3-7 Intracellular Ca2+ concentration increases after OA stimulation

Calcium flux detected by R47A04-Gal4 driving UAS-GCaMP5G in mature follicle cells. The zero time point is 15 seconds before OA administration. The signal intensity was maximum around 6:30 (mm:ss). Fluorescence intensities are presented using a false-color scale, shown in the first panel.

Supplemental Figure 3-8 Mmp2::GFP fusion protein is trapped intracellularly

(A-A') GFP antibody is applied after the fixation to permeabilize the cell membrane. Mmp2::GFP is detected in posterior follicle cells. (B-C') GFP antibody is applied before the fixation to label the
extracellular Mmp2::GFP. Mmp2::GFP is not detected in posterior follicle cells without (B-B') and with (C-C') OA stimulation. Together with the fact that Mmp2::GFP homozygous females are lethal, this result indicate that Mmp2::GFP fusion proteins are trapped inside the cell.

### Table 1. The effect of follicular adrenergic signaling on egg laying, egg distribution in the reproductive tract, and egg laying time.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Egg laying in 2 days&lt;sup&gt;6&lt;/sup&gt;</th>
<th>Egg distribution in 6h</th>
<th>Egg laying time (min)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>N Eggs/ female/ day</td>
<td>N Uterus with egg (%)</td>
<td>Total time</td>
</tr>
<tr>
<td>Ovar&lt;sup&gt;TM3217/TM3&lt;/sup&gt;</td>
<td>20</td>
<td>59.3 ± 2.8</td>
<td>76</td>
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<tr>
<td>UAS-dcr2/++; 44E1-10-Gal4/+ (Ore-R)</td>
<td>20</td>
<td>5.9 ± 3.6***</td>
<td>61</td>
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<tr>
<td>UAS-dcr2/++; 44E1-10-Gal4/+ ( Attp2)</td>
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<td>57.2 ± 2.7</td>
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<tr>
<td>UAS-dcr2/++; 44E1-10-Gal4/+ (Gambia)</td>
<td>50</td>
<td>72.4 ± 2.0**</td>
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<td>UAS-dcr2/++; 44E1-10-Gal4/+ (Gambia/4)</td>
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<td>11.4 ± 1.2***</td>
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<td>UAS-dcr2/++; 44E1-10-Gal4/+ (Gambia/3)</td>
<td>25</td>
<td>21.3 ± 1.5***</td>
<td>76</td>
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<tr>
<td>UAS-dcr2/++; 44E1-10-Gal4/+ (Gambia/2)</td>
<td>50</td>
<td>27.8 ± 2.2***</td>
<td>120</td>
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<td>UAS-dcr2/++; 44E1-10-Gal4/+ (Gambia/1)</td>
<td>25</td>
<td>31.0 ± 4.2**</td>
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<td>UAS-dcr2/++; 47A04-Gal4/+ (Ore-R)</td>
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<td>71.4 ± 1.6</td>
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<td>UAS-dcr2/++; 47A04-Gal4/+ (Gambia)</td>
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<td>29.3 ± 2.3***</td>
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<td>UAS-dcr2/++; 47A04-Gal4/+ (Gambia/2)</td>
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<td>44.5 ± 1.5***</td>
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<td>UAS-dcr2/++; 47A04-Gal4/+ (Gambia/3)</td>
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<td>53.5 ± 2.5**</td>
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<td>UAS-dcr2/++; 47A04-Gal4/+ (Gambia/4)</td>
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<td>63.0 ± 3.4</td>
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# one day = 22h at 29°C
* P<0.05
** P<0.01
*** P<0.001
doi:10.1371/journal.pgen.1005604.001

### Table 2. The effect of follicular adrenergic signaling for follicle trimming.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Mated or Virgin</th>
<th>No. of females</th>
<th>Mature eggs / female</th>
<th>Posterior trimmed eggs / female</th>
<th>Normalized trimming eggs / female (%)</th>
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<tbody>
<tr>
<td>UAS-dcr2/++; 44E1-10-Gal4/ + (Ore-R)</td>
<td>6 hr mating</td>
<td>30</td>
<td>40.3 ± 21.5</td>
<td>3.7 ± 1.9</td>
<td>9.0 ± 3.9</td>
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<tr>
<td>UAS-dcr2/++; 44E1-10-Gal4/ Gambia/4</td>
<td>6 hr mating</td>
<td>29</td>
<td>43.1 ± 12.1</td>
<td>1.4 ± 0.9***</td>
<td>3.1 ± 1.9***</td>
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<tr>
<td>TDC&lt;sub&gt;Ros&lt;/sub&gt; / Cyo</td>
<td>6 hr mating</td>
<td>56</td>
<td>10.4 ± 11.6</td>
<td>1.3 ± 1.2</td>
<td>15.0 ± 16.5</td>
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<tr>
<td>TDC&lt;sub&gt;Ros&lt;/sub&gt; / DGR42, cn1</td>
<td>6 hr mating</td>
<td>47</td>
<td>35.5 ± 11.6*</td>
<td>0.2 ± 0.4***</td>
<td>0.4 ± 1.1***</td>
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<td>ThH&lt;sub&gt;11&lt;/sub&gt; / FM7</td>
<td>6 hr mating</td>
<td>20</td>
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<td>2.6 ± 1.3</td>
<td>21.4 ± 22.9</td>
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<td>ThH&lt;sub&gt;11&lt;/sub&gt; / FM7</td>
<td>6 hr mating</td>
<td>20</td>
<td>29.0 ± 8.4*</td>
<td>0.8 ± 1.2***</td>
<td>2.4 ± 3.6***</td>
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<td>ThH&lt;sub&gt;11&lt;/sub&gt; / FM7</td>
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<td>20</td>
<td>35.3 ± 6.4</td>
<td>6.5 ± 2.7</td>
<td>18.6 ± 7.6</td>
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<td>ThH&lt;sub&gt;11&lt;/sub&gt; / FM7</td>
<td>Virgin</td>
<td>20</td>
<td>43.8 ± 11.5**</td>
<td>1.0 ± 1.2***</td>
<td>2.0 ± 2.8***</td>
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* P<0.05
** P<0.01
*** P<0.001
doi:10.1371/journal.pgen.1005604.002
S1 Table. The analysis of kinetics of ex vivo follicle rupture.

Table S1. The analysis of kinetics of ex vivo follicle rupture

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<th>Experiments</th>
<th>Total follicles for recording</th>
<th>total ruptured during recording</th>
<th># follicle for analysis</th>
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<th>Finishing time (min)</th>
<th>Rupture time (min)</th>
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a. One follicles initiate rupture before recording.
b. Two follicles initiate rupture before recording.
c. The same as Movie S1.
d. Defined as the time frame when 5% of posterior tip without follicle-cell cover.
e. Defined as the time frame when more than 95% of oocytes without follicle-cell cover.
f. Follicles were isolated from five females for each experiment.
CHAPTER 4: STORE-OPERATED CALCIUM ENTRY FUNCTIONS IN MATURE FOLLICLE CELLS FOR DROSOPHILA OVULATION

INTRODUCTION

Adrenergic signaling regulates many diverse aspects of physiology across Animalia despite only having two neurohormones to communicate: norepinephrine/epinephrine (vertebrates) or octopamine/tyramine (invertebrates). Diversity in response to the signal is achieved by the receptors on the target tissue, which in the case of adrenergic signaling are G-protein coupled receptors (GPCRs). The monoamine octopamine is of wide interest in the invertebrate field due to its involvement in a wide variety of behaviors in many different model organisms. Drosophila have four octopamine receptors: Oamb, Octβ1R, Octβ2R, Octβ3R (reviewed in (El-Kholy et al., 2015)). Despite the universality of octopaminergic signaling in Drosophila, the signal transduction pathways for its receptors remain undescribed.

In particular, Oamb is involved in many behaviors and physiological systems in Drosophila, such as feeding (Branch et al., 2017; Burke et al., 2012; Huetteroth et al., 2015; Kim et al., 2013; Luo et al., 2014), aggression (Watanabe et al., 2017), courtship conditioning (Zhou et al., 2012), sleep (Crocker et al., 2010), and ovulation / egg transport (Deady and Sun, 2015; Lee et al., 2003b, 2009). Oamb activation by octopamine has been demonstrated to increase cAMP accumulation in Drosophila S2 cells (Han et al., 1998) and dILP neurons (Crocker et al., 2010); in contrast, calcium concentration is increased upon octopamine stimulation in HEK cells (Han et al., 1998), 0104 Gal4 labeled neurons (Burke et al., 2012), and stage-14 follicle cells (Deady and Sun, 2015; Deady et al., 2017).

Drosophila are an outstanding model system to study ovulation due to physiological and genetic conservation, for example: a requirement for matrix metalloproteinases (Deady et al., 2015), steroid hormone signaling (Knapp & Sun, 2017), adrenergic signaling (Deady and Sun, 2015), and reactive oxygen species production (Li et al., In review). Similar to mammals,
*Drosophila* have two ovaries, which are connected to the uterus by their respective oviducts. Egg chambers develop throughout 14 distinct stages (Spradling, 1993) to ultimately produce a fertilizable oocyte. Octopamine is indispensable for female fertility in *Drosophila*; both ovulation, expulsion of a mature oocyte from its follicle cell layer (follicle rupture), and egg transport from the ovary to the uterus rely heavily on octopaminergic signaling (Cole et al., 2005; Deady and Sun, 2015; Lee et al., 2003b, 2009; Lim et al., 2014; Middleton et al., 2006). Oct2βR and Oamb activation on the oviduct epithelium causes the oviduct musculature to relax, allowing for the oocyte to travel from the ovary to the uterus (Lee et al., 2003; Lee et al., 2009; Lim et al., 2014). The relaxation of the oviduct was hypothesized to allow for the oocyte to passively leave the ovary and be transported to the uterus. A more recent demonstration of octopamine’s function in ovulation involves octopamine acting in the ovaries; Oamb activation on stage-14 follicle cells increases intracellular calcium levels and induces follicle rupture (Deady and Sun, 2015). Although increased calcium is both essential and sufficient to induce follicle rupture, the signal transduction of Oamb on stage-14 follicle cells to induce calcium increase remains unknown.

In this study, we describe the signal transduction of Oamb in Drosophila stage-14 follicle cells. Oamb acts through Gαq/IP3-mediated signal transduction to increase intracellular calcium concentration. Through development of an *ex vivo* calcium imaging assay, we demonstrate that depletion of calcium stores induces store-operated calcium entry to further increase calcium concentration, ultimately resulting in follicle rupture and ovulation.

**RESULTS**

**Gαq-mediated signal transduction is essential and sufficient for follicle rupture**

The signal transduction of Oamb has remained a mystery since its discovery twenty years ago and the G proteins that transduce the Oamb signal are unknown. In stage-14 follicle cells, Oamb activation leads to an increase of intracellular calcium (Deady and Sun, 2015; Deady et al., 2017) indicating Gαq might be the G-protein to transduce the Oamb signal. To test this
hypothesis, RNAi was used to deplete $G_{\alpha q}$ expression in stage-14 follicle cells. If $G_{\alpha q}$ is coupled to Oamb, females expressing follicle-cell specific $Gaq$-RNAi (simplified as $G_{\alpha q}$-RNAi females) should phenocopy $Oamb$-RNAi females. (Deady and Sun, 2015). When assayed for ovulation ability in vivo, $G_{\alpha q}$-RNAi females displayed a significant decrease in egg laying (Figure 1A and D), which was not due to an oogenesis defect as these females had a comparable number of mature follicles present in their ovaries post egg-laying (Figure S1A and B). Furthermore, $G_{\alpha q}$-RNAi females had significantly increased estimated ovulation time (Figure 1B and E). Their incompetency to ovulate was also observed in our ex vivo follicle rupture assay, in which mature follicles were isolated and stimulated with octopamine to induce rupture (Figure 1C and F – filled bars). In contrast, follicles were mostly competent to rupture in response to ionomycin stimulation (Figure 1C and F – open bars). These phenotypes manifested using two independent RNAi lines and two independent follicle-cell specific Gal4 drivers, “FC1-Gal4” and “FC2-Gal4” (Deady et al., 2017). Interestingly, these results phenocopy $Oamb$-RNAi follicles, wherein they show decreased ovulation, decreased response to octopamine-stimulated follicle rupture, while are competent to rupture in response to ionomycin stimulation.
Figure 4.1 Gαq is essential and sufficient for follicle rupture

Egg laying capacity (A, D) and estimated ovulation time (B, E) in follicles with Gαq-RNAi. Octopamine-stimulated (C and F, filled bars) and ionomycin-stimulated (C and F, open bars) follicle rupture in follicles with Gαq-RNAi; data are plotted as percent of ruptured follicles. Data from FC1 Gal4 is plotted in black bars and FC2 Gal4 is plotted in grey bars. Number of females (egg-laying) or number of replicates (ex vivo cultures) are listed above each bar. Overexpression of constitutively-active Gαq induces precocious rupture (G-J). DAPI (nuclei) is shown in blue/black and egg chamber autofluorescence (FITC) in green are shown in G-I. Overexpression of constitutively-active Gαq mutation GaqQ203L induces ectopic follicle rupture within the ovary when driven by FC1 (G-H) or FC2(I) Gal4 drivers. Precocious rupture phenotype quantification (J); the number of ovaries analyzed is above each bar; one data point is one ovary and overlaid is average ± SD. *p < 0.05, **p < 0.01, ***p < 0.001.
To determine whether activation of Gαq was sufficient to induce ovulation, a dominant-active form of Gαq (Gαq$^{Q203L}$, (Ratnaparkhi et al., 2002)) was overexpressed in mature follicle cells. Interestingly, follicles with Gαq$^{Q203L}$ overexpression exhibited a precocious rupture phenotype. Oocytes in control ovaries were always surrounded by a layer of follicle cells (Figure 1G and J). In contrast, stage-14 egg chambers expressing the Gαq-overactive mutation contained a mature oocyte which was completely uncovered and the follicle-cell layer had receded toward to the dorsal-appendages (Figure 1H, J, K), and this was never observed in control egg chambers. The only example of oocytes being exposed (i.e. without follicle-cell envelopment) within the ovary is follicle-cell trimming, wherein the posterior few follicle cells open and begin to squeeze toward the anterior while ejecting the oocyte toward the oviduct. (Deady et al., 2015). Because the follicles which were completely receded maintained follicle-cell specific expression of RFP (Figure S2A and B), we characterize this phenotype as “precocious rupture”. When assayed for egg-laying ability, females with Gaq$^{Q203L}$ follicles laid only ~5 eggs per female per day compared to control females which laid ~50 eggs per day (Figure S2C), indicating that precocious rupture is detrimental to fertility. Altogether, these results demonstrate Gαq is essential for ovulation and its activation is sufficient to induce follicle rupture.

**IP3-receptor is essential for ovulation**

Canonical Gαq signal transduction results in IP3 generation and subsequent binding to IP3 receptors (IP3R, encoded by Itpr83a) on the endoplasmic reticulum. IP3R activation results in release of calcium from endoplasmic reticulum stores and therefore increased cytoplasmic calcium levels. To address whether IP3/IP3R signaling was indeed also involved in octopamine signaling in stage-14 follicle cells, we used RNAi to knock-down Itpr83a (Simplified as “IP3R-RNAi”) and assay ovulation capacity.

Similar to Gaq-RNAi, when females with IP3R-RNAi were assayed for ovulation ability in
vivo, egg laying was significantly reduced (Figure 2A and E), which was not attributable to an oogenesis deficiency (Figure S1C and D). This decrease in ovulation capacity was accompanied by a significant increase in estimated ovulation time (Figure 2B and F). To determine whether the in vivo ovulation phenotype was attributable to defective follicle rupture, follicles were isolated and stimulated with octopamine or ionomycin in the ex vivo to directly assay follicle rupture competency. Octopamine was unable to stimulate follicle rupture (Figure 2C and G) in follicles expressing IP3R-RNAi, whereas follicles were generally competent to rupture in response to ionomycin (Figure 2D and H). These phenotypes manifested using two independent RNAi lines and two independent follicle-cell specific Gal4 drivers. Altogether these data demonstrate a functional requirement for IP3R in follicle rupture downstream of octopaminergic stimulation and upstream of intracellular calcium increase.

**Figure 4.2 IP3R is required for ovulation**

Egg laying capacity (A, E) and estimated ovulation time (B, F) in follicles with IP3R-RNAi. Octopamine-stimulated (C, G) and ionomycin-stimulated (D, H) follicle rupture in follicles with IP3R-RNAi; data are plotted as percent of ruptured follicles. FC1 Gal4 is plotted in black bars and FC2 Gal4 is plotted in grey bars. Number of females (egg-laying) or number of replicates (ex vivo cultures) are listed above each bar. *p < 0.05, **p < 0.01, ***p < 0.001.
Store-operated calcium entry is required for follicle rupture

To determine whether extracellular calcium was required for octopamine-induced rupture, we performed an ex vivo rupture assay with calcium-chelated media (with BAPTA). When cultured with BAPTA at concentrations of 5 mM, 10 mM, or 20 mM, octopamine-induced rupture was significantly reduced, whereas 1 mM BAPTA or control media containing sucrose, octopamine was still sufficient to induce rupture (Figure 3A).

![Image](image.png)

Figure 4.3 Store-operated calcium entry is required for normal ovulation

Response of control follicles (FC2 > +) to octopamine-induced rupture when cultured with BAPTA (A). Expression of calcium channels in stage-14 egg chambers (black bars; microarray “arbitrary units”) or stage 13/14 egg chambers (blue bars; RNA-seq “RNA(RPKM)”; see
Materials & Methods). Egg laying capacity (C, G) and estimated ovulation time (D, H) in follicles with Stim-RNAi. Octopamine-stimulated (E, I) and ionomycin-stimulated (F, J) follicle rupture in follicles with Stim-RNAi; data are plotted as percent of ruptured follicles. FC1 Gal4 is plotted in black bars and FC2 Gal4 is plotted in grey bars. Number of females (egg-laying) or number of replicates (ex vivo cultures) are listed above each bar. *p < 0.05, **p < 0.01, ***p < 0.001.

Reduction of octopamine-induced rupture with decreased external calcium available suggests a role for external calcium in follicle rupture. If extracellular calcium is a requisite for octopamine-induced rupture and calcium increase, then there must be a mode for calcium entry to the cytoplasm. To determine through which channels may be contributing to calcium entry, two recent datasets were examined (Kronja et al., 2014; Tootle et al., 2011) to identify relative levels of a suite of genes in stage-14 egg chambers that could be required for calcium-entry from the external milieu (Figure 3B; see Materials and Methods). Stim (stromal interaction molecule) was the only gene that had consistently high expression in stage-14 egg chambers from both data sets, therefore we decided to investigate Stim further. In brief, Stim is activated upon depletion of calcium from endoplasmic reticulum stores. Active Stim interacts with membrane channel Orai to form a complex which forms a pore allowing for calcium influx (reviewed in (Soboloff et al., 2012)).

To determine whether Stim was integral in mature follicle cells for ovulation, we used RNAi to deplete the expression of Stim in stage-14 follicle cells. Stim-RNAi females exhibited significantly reduced egg laying (Figure 3C and G) despite normal oogenesis (Figure S1E and F). This was accompanied by increased estimated ovulation time (Figure 3D and H). Consistent with the in vivo ovulation phenotype, Stim-RNAi females showed severe reduction to octopamine-induced rupture compared to controls (Figure 3E and I), demonstrating Stim is essential for follicle rupture. Despite the lack of competency to rupture in response to octopamine, Stim-RNAi follicles were fully competent to rupture in response to ionomycin (Figure 3F and J). These phenotypes manifested using two independent RNAi lines and two independent follicle-cell specific Gal4 drivers. These results demonstrate that Stim functions downstream of octopamine stimulation and
upstream of calcium to regulate follicle rupture.

**Calmodulin/CaMKII is a downstream target of Oamb**

We sought to determine a potential downstream target of increased calcium signaling. Therefore, we decided to investigate calmodulin (Cam) and calcium/calmodulin-dependent protein kinase (CaMKII). Females expressing RNAi against *Cam* or *CaMKII* (simplified as “*Cam-RNAi*” or “*CaMKII-RNAi*”) were assayed for ovulation ability *in vivo*. There was a significant decrease in egg laying (Figure S3A and E), which was not attributable to an oogenesis deficiency (Figure S1G and H) and was accompanied by a significant increase in estimated ovulation time (Figure S3B and F). Furthermore, follicles were extracted and stimulated with octopamine or ionomycin *ex vivo* to directly assay follicle rupture. Octopamine was unable to stimulate follicle rupture (Figure S3D and G). Follicles expressing *Cam-RNAi* or *CaMKII-RNAi* earlier with FC1-Gal4 (stages 14A-C) were incompetent to rupture in response to ionomycin stimulation, which was suggestive of a role of downstream of calcium increase. Follicles expressing *Cam-RNAi* or *CaMKII-RNAi* later (stage 14C) with FC2-Gal4 were mostly competent to rupture in response to ionomycin (Figure S3D and H). While these experiments only demonstrate a role for one potential target of calcium signaling, they demonstrate an essential role for *Cam* and *CaMKII* in follicle rupture downstream of octopaminergic and intracellular Calcium increase.

**Development of ex vivo calcium imaging assay**

To examine the mobilization of calcium more directly, we developed an *ex vivo* calcium imaging assay. Follicles expressing *GCaMP6f* (Chen et al., 2013) were isolated and changes in fluorescence intensity, an indicator of calcium concentrations, were measured (Figure 4A). When stimulated with 0.5 or 1 μM octopamine, follicles exhibited a cycling wave of increased calcium consistently returning to baseline throughout the ~30-minute recording (Figure S4A and B). At higher concentrations of octopamine (5, 20 μM), follicles exhibited a robust increase in intracellular calcium concentration, which had a characteristic large peak within ~two minutes, a
decay (varying in both duration and magnitude) in signal, and a subsequent maintenance of high signal (Figure S4C; Figure 4C and I). When stimulated with ionomycin, control follicles exhibited a gradual increase in signal that maintained high throughout the experiment (Figure 4D and J) which was highly variable, likely due to the rate of diffusion in the media. When stimulated with thapsigargin at 5, 10, and 20 μM (to release internal stores of calcium and inhibit SERCA channels) follicles exhibited an increased signal that was slow to decay (Figure 4E and K, Figure S5), demonstrating release of ER-calcium stores induces a prolonged, sustained calcium signal. Furthermore, thapsigargin was sufficient to induce follicle rupture in control follicles (Figure S5D). These data demonstrate that octopamine, as well as ionomycin and thapsigargin, can stimulate an increase in follicular calcium concentrations. To confirm that external calcium was required for octopamine-induced calcium increase, control follicles were cultured in calcium-chelated media (BAPTA). When cultured in BAPTA, every aspect of octopamine-induced calcium increase was dramatically altered compared to sucrose controls (Figure 4G, M-O). In each concentration of BAPTA tested, a minimal peak was observed when stimulated with octopamine and this signal was not maintained. This trend was also observed when thapsigargin was used to stimulate increased calcium concentrations (Figure 4P). In contrast, sucrose controls exhibited a substantial initial increase in GCaMP signal in response to octopamine stimulation, followed by a slight decrease in signal, and was ultimately maintained at high levels (Figure 4F, L), similar to culture media only controls. Area-under-the-curve (AUC) analysis demonstrates comparable calcium levels in octopamine-, ionomycin-, or thapsigargin-stimulated follicles, and in contrast, AUC is severely reduced in BAPTA (Figure 4Q). These results highlight the significance of external calcium for octopamine induced calcium increase.
Figure 4.4 Octopamine-induced calcium increase requires calcium influx

Schematic of *ex vivo* calcium imaging set-up (A). Representative still-frames from GCaMP recordings are shown at 0, 5, 10, 20, and 30 minutes for control follicles (B), follicles stimulated with octopamine ("OA", C), ionomycin (D), thapsigargin (E), 50 mM sucrose + octopamine (F), 10 mM BAPTA + octopamine (G), and 10 mM BAPTA + thapsigargin (H). One representative
video was chosen wherein the response from each follicle in that video is plotted in grey and the average from that video is overlaid in color. The scale bar is 100% ΔF/F0 (vertical) and 5 minutes (horizontal). At T= ~3 minutes, octopamine (black arrow; I, L-O), ionomycin (red arrow; J), or thapsigargin (orange arrow; K, P) was added. The area under the curve (“AUC”) average is plotted and the value for every follicle recorded is overlaid in colored circles (Q). Number of egg chambers analyzed is above each bar. Every follicle in this figure is wild-type.

**Oamb and Stim are required for normal octopamine-induced calcium increase**

We aimed to utilize the newly developed *ex vivo* calcium imaging assay to confirm genes which are speculated to function in the Oamb pathway. Firstly, we sought to demonstrate that Oamb stimulation by octopamine indeed results in increased intracellular calcium. Follicles expressing both *GCaMP6f* and *Oamb-RNAi* were cultured and stimulated with octopamine. As expected, follicles with *Oamb-RNAi* had a prominently attenuated response than controls; although there was an initial increase in signal in response to octopamine, high GCaMP levels (Figure 5A and E) were not sustained. This phenotype is exemplified by AUC analysis (Figure 5K). Despite the lack of response to octopamine, follicles expressing both *GCaMP6f* and *Oamb-RNAi* were able to increase GCaMP signal to around control levels when stimulated with thapsigargin (Figure 5F) or ionomycin (Figure 5G).
Figure 4.5 Oamb and Stim are required for octopamine-induced SOCE

Representative still-frames from GCaMP recordings are shown at 0, 5, 10, 20, and 30 minutes for follicles expressing Oamb-RNAi (A) or Stim-RNAi (B-D). One representative video was chosen wherein the response from each follicle in that video is plotted in grey and the average from that video is overlaid in blue (for Oamb-RNAi, E-G) or purple (for Stim-RNAi, H-J). The scale bar is 100% ΔF/F0 (vertical) and 5 minutes (horizontal). At T= ~3 minutes, octopamine (“OA”, 20 μM, E and H), thapsigargin (“Tg”, 10 μM, F and I), or ionomycin (“iono”; 5 μM, G and J) was added. The area under the curve (“AUC”) average is plotted and the value for every follicle recorded is overlaid in colored circles (K). Number of egg chambers analyzed is above each bar.
To confirm that Stim was also required for octopamine-induced increased calcium, follicles expressing Stim-RNAi were assayed for their ability to increase calcium levels. These follicles had a severe reduction in octopamine-induced and thapsigargin-induced calcium increase (Figure 5B, C, H and I) compared to controls (Figure 4C, E, I, L), however exhibited a robust increase when stimulated with ionomycin (Figure 5D and J). These observations are also demonstrated by AUC analysis (Figure 5K). This dramatic attenuation in calcium increase in Stim-RNAi follicles when Oamb-induced (by octopamine) or endoplasmic reticulum-induced (by thapsigargin) calcium increase demonstrate the majority of calcium signal is likely attributed to inability to trigger influx of external calcium. As expected as a downstream target of calcium signaling, RNAi-depletion of CaMKII in follicle cells does not perturb octopamine-induced calcium increase (Figure S6).

**Octopaminergic neurons stimulate Oamb to generate follicular calcium waves in vivo**

In attempt to determine whether octopamine induces increased intracellular calcium *in vivo*, we developed an assay to visualize calcium signaling in follicle cells in an intact fly. A follicle-cell specific Gal4 driver was used to express a genetically encoded calcium indicator, GCaMP6f, to observe spontaneous changes in calcium concentrations in follicle cells through her ventral abdomen (Figure 6A) while tethered to a glass slide. In intact control females, spontaneous waves of calcium increases were observed throughout the main-body follicle cells and follicle cells surrounding the dorsal appendages (Figure 6B, C, E, G, I, L). In attempt to determine the possible role of octopamine in these spontaneous calcium waves, we used a TβH<sup>M18</sup> mutant (females unable to synthesize octopamine) or silenced octopaminergic neurons (TDC2>Kir2.1) and assayed spontaneous activity. When octopaminergic signaling was perturbed, no spontaneous waves were observed throughout the main-body follicle cells (Figure 6D, F, and L). These results demonstrate that octopamine induces spontaneous waves of increased intracellular calcium throughout mature follicle cells.
Figure 4.6 Octopamine stimulates calcium waves through Oamb in vivo

Experimental set-up for in vivo GCaMP imaging through the female abdomen (A). The pink dashed line is a horizontal line (~1 mm) drawn horizontally across the female abdomen and corresponds with the x-position measured for the kymographs. Representative images of a control follicle throughout ~2 minutes of recording; time (min:sec) for each still-frame is noted at the upper-right corner of each panel (B). Kymographs from representative videos of females from the following genotypes: TβH<sup>M18</sup>/+ (C), TβH<sup>M18</sup> (D), TDC2;++ (E), TDC2>Kir2.1 (F), or Oamb<sup>-/-</sup> (G), Oamb<sup>-/-</sup> (H), FC++ (I), FC>Oamb-RNAi (J), or FC>Stim-RNAi (K) females. Quantification of the phenotypes; percent is listed with the number of females in parentheses (L).

We aimed to confirm whether Oamb was indeed the receptor required for octopamine-induced calcium waves. Spontaneous calcium waves were not observed in the main-body follicle cells in females which are mutant for the Oamb receptor (Oamb<sup>-/-</sup>; Figure 6H and L). Furthermore, the majority of females with follicular Oamb-RNAi did not have spontaneous calcium signals in the main-body follicle cells (Figure 6J and L), demonstrating follicular Oamb expression is required for spontaneous calcium waves in vivo. Interestingly stretch follicle cells surrounding the dorsal appendages still maintained spontaneous calcium waves in both Oamb<sup>-/-</sup> and Oamb-RNAi (Figure 6L). These experiments demonstrate octopamine induces Oamb to activate increased
intracellular calcium in stage-14 main-body follicle cells \textit{in vivo}. Lastly, we wanted to confirm the role for Stim in regulating octopamine-induced calcium waves \textit{in vivo}. \textit{Stim-RNAi} was coexpressed with \textit{GCaMP6f} and females were imaged. No spontaneous calcium signals were observed in ~50% of females tested, whereas brief flashes of increased fluorescence were observed in the other ~50% of females (Figure 6K, L). These results suggest store-operated calcium entry functions in spontaneous calcium waves \textit{in vivo}.

\textbf{DISCUSSION}

In this study, we sought to determine the signal transduction pathway of octopaminergic activation of Oamb in stage-14 follicle cells. We identified Gaq signal transduction is both essential and sufficient for follicle rupture (Figure 1). We also identified the IP3-receptor is also imperative in this pathway (Figure 2). Internalization of extracellular calcium via Stim is critical in mediating follicle rupture and normal octopamine-induced increase in calcium (Figures 3, 4, 5). Furthermore, we demonstrated spontaneous calcium waves \textit{in vivo} occur in the stage-14 follicle cells, and they are octopamine, Oamb, and Stim dependent (Figure 6). The results from this study describe the signal transduction of Oamb to ultimately result in follicle rupture. Although we cannot completely rule out Gaq may be interacting with another GPCR in the stage-14 follicle cells, we are confident that it is functionally downstream of octopaminergic stimulation and upstream of calcium signaling. Furthermore, this Oamb is the only octopamine-activated receptor that is expressed in stage-14 follicle cells (Deady and Sun, 2015).

\textbf{Store-operated calcium entry in epithelial cells}

The concept of calcium influx triggered by depletion of ER-stores of calcium was first described in 1989 (Takemura and Putney, 1989). The two major players of SOCE, Stim and Orai were characterized in 2005 (Liou et al., 2005; Roos et al., 2005) and 2006 (Prakriya et al., 2006; Yeromin et al., 2006), respectively. Humans have two Stim genes and three Orai genes (\textit{Stim1},
Stim2, Orai1, Orai2, Orai3), whereas Drosophila have one of each (Stim and Olf-186-f). 
Drosophila have already been demonstrated to be an apt model to study SOCE; an example of functional conservation between species is a requirement for normal Stim function for fat storage in both humans (Shi et al., 2000) and flies (Baumbach et al., 2014).

Despite its universal importance, calcium signaling in epithelial cells is not well studied in live tissue. Development and improvement of genetic tools, including pharmacogenetics, have allowed for exceptional temporal and spatial resolution in interrogating pathways of interest. Recently, work in the Drosophila wing disc has demonstrated intercellular calcium waves occurring amongst sheets of epithelial cells (Balaji et al., 2017) which can be stimulated by incubation in fly extract or ovary extract, and this was through store-operated calcium entry. However, this phenomenon was unable to be observed in vivo. This study provides for the first time a model system, the Drosophila ovaries, wherein genetic manipulation can be made and resultant changes in epithelial calcium concentrations can be directly measured in real-time.

Sustained level of calcium leads to follicle rupture

Here, we demonstrate that the majority of octopamine-induced calcium increase is attributable to extracellular calcium. Chelating extracellular calcium in a three-hour culture substantially reduces the rupture response to octopaminergic stimulation (Figure 3A). Even more dramatically, almost no calcium signal was observed when extracellular calcium was chelated during the ~30-minute ex vivo recording (Figure 3G, L-N). It is interesting to speculate that the observed brief, small increase (~10% ΔF/F0) of follicles in BAPTA-containing media could be demonstrating internal stores of calcium (Figure 3L and M) and the remainder of the signal is from external stores. This trend is evident in octopamine-stimulated follicles in BAPTA (Figure 4G, M-O), thapsigargin-stimulated follicles in BAPTA (Figure 4H, P), and octopamine- or thapsigargin stimulated Stim-RNAi follicles (Figure 5B-D, H, I). Altogether these results demonstrate the
essential role for store-operated calcium entry for a normal octopamine-induced calcium increase.

Low concentrations of octopamine (1 μM) induce an initial peak of calcium increase that is comparable to higher concentrations (≥5 μM) but are typically followed by a cycling wave which continuously returns to baseline. We previously described a dose-response relationship between octopamine concentration and follicle rupture (Deady and Sun, 2015). In those experiments, 1 μM octopamine was insufficient to induce follicle rupture, whereas concentrations at or above 5 μM were able to induce rupture, which a stable maximum response at 20 μM octopamine. Interestingly, in vivo we observed spontaneous waves of calcium increases throughout the follicle cells, however we did not observe sustained main-body follicle cell signal nor spontaneous follicle rupture / ovulation. Therefore, we propose that follicle rupture occurs when a locally high (>1 μM) concentration of octopamine induces sustained levels of increased calcium concentrations likely through a SOCE-mediated process.

It is exciting to speculate what the function is of the increased, sustained level of calcium within the follicle cells to ultimately cause follicle rupture. Increased levels of epithelium calcium concentrations are linked to apoptosis, transcription, and actin/myosin kinetics. The follicle cells remain in their same anterior-posterior orientation and retain expression of markers, such as hindsight and ecdysone-synthesis enzymes (Deady et al., 2017; Knapp and Sun, 2017) within the ovary after the oocyte is ovulated. A particularly attractive idea would be a coordination of follicle cell shape changes or actin-myosin contraction to actively expel the oocyte during follicular rupture, however this remains unknown.

**Follicle rupture is a conserved physiological process**

Ovulation depends on temporally- and spatially- appropriate follicular rupture. In mice, LH/LHR induces increased expression of progesterone receptor, which coordinates the upregulation of a suite of genes required for follicle rupture, for example ADAMTS1 (Brown et al.,
Female mice null for PGR, whose expression is induced by LHR, or ADAMTS-1, induced by PGR, are unable to rupture fully developed follicles and interestingly Gαq/11 phenocopies these mice (Breen et al., 2013; Brown et al., 2010; Robker et al., 2009)), demonstrating a role upstream of the progesterone receptor for Gαq in regulating follicle rupture. In this study we demonstrate conservation for the role of Gαq in follicle rupture in the somatic cells surrounding the oocyte. In Drosophila, Gαq is both essential and sufficient for follicle rupture and is most likely downstream of Oamb in its signal transduction. This study provides yet another instance of genetic conservation between Drosophila and mammals.

MATERIALS AND METHODS

Drosophila genetics

Flies were reared in 25 °C on a standard diet consisting of cornmeal and molasses. All experiments using RNAi expressed UAS-dcr2 and were performed at 29 °C. See the supplemental table for the list of genotypes used for each figure. The following RNAi lines were acquired from the Vienna Drosophila Resource Center: Gaq-RNAi (#50729), Gaq-RNAi (#105300), Itpr83a-RNAi (#6486), Itpr83a-RNAi (#106982), Stim-RNAi (#47073), Stim-RNAi (#106256), Oamb-RNAi (#2681), CaMKII-RNAi (#47280). The following Gal4 / LexA drivers were from the Janelia collection (R44E10-Gal4 “FC1”, R47A04-Gal4 “FC2”, R47A04-LexA, LexAOp-GCaMP6f,(Chen et al., 2013; Pfeiffer et al., 2008). Both follicle-cell specific Gal4 drivers were recombined with UAS-RFP to identify and isolate mature follicle cells. The following stocks were acquired from the Bloomington Drosophila Stock Center: GaqQ203L (BDSC # 30743, (Ratnaparkhi et al., 2002), TDC2-Gal4 (BDSC # 9313,(Cole et al., 2005)), Oamb(Df) (BSC141 - BDSC # 9501,(Parks et al., 2004)), Calmodulin-RNAi (BDSC # 34609), tub-Gal80ts (BDSC # 7019). TβhM18 (Monastirioti, 2003) was provided from Dr. Mariana Wolfner, UAS-Kir2.1 ((Hardie et al., 2001)–
Sean Sweeney donor was provided from Dr. Benjamin White, *Oamb<sup>M12417</sup>* (Venken et al., 2011), and *UAS-Mmp2-RNAi* was from (Uhlirova and Bohmann, 2006).

**Ovulation assays**

*in vivo ovulation assays:* Egg laying, mature egg analysis, and egg distribution have been described previously. (Deady and Sun, 2015; Deady et al., 2015, 2017; Knapp and Sun, 2017; Sun and Spradling, 2013) In brief, the egg laying experiment consists of five females and ten Oregon-R males are mated for 22 hours per day in 29 °C and females are allowed to lay eggs on a molasses plate substrate lined with wet yeast paste. One data point is the average number of eggs from one bottle (five females) per one day. Data are displayed as the average number of eggs per female ± SEM. After two days of the egg laying experiment, ovaries are dissected intact from females, fixed in paraformaldehyde, and their mature follicles are counted (identified by autofluorescent dorsal appendages through the FITC filter). Data are displayed as the average number of follicles per female ± SD. To calculate estimated ovulation time, first the total time for a female to lay one egg is calculated, data extrapolated from the egg laying experiment. Then, that time is partitioned into: amount of time the egg is ovulating / transporting the egg / withholding in the uterus (data extrapolated from an egg location assay wherein six-hour mated females are dissected and the distribution of an egg throughout the reproductive tract is noted).

*ex vivo ovulation assays:* A protocol detailing ex vivo follicle rupture was recently published (Knapp et al., 2018). In brief, fluorescently labeled stage-14 follicles were isolated and stimulated with octopamine (20 μM) or ionomycin (5 μM) or thapsigargin to induce follicle rupture. One data point represents one group (25-35 follicles) as the percent of follicles that ruptured throughout the span of the culture. For BAPTA experiments, culture media was prepared the day of the experiment with the desired concentration of BAPTA added. Control experiments used culture media with sucrose prepared in the same way. Average percent of ruptured follicles is plotted, ± SD.
**GCaMP imaging**

*ex vivo*: R47A04-LexA, LexAOp-GCaMP6f; R44E10-Gal4, UAS-dcr2, Oamb-RFP* virgins crossed to control or RNAi males (*R47A04>*GCaMP to observe calcium signals; *R44E10>*UAS-RNAi to knock down genes of interest; *Oamb-RFP* to select mature follicles). Follicles from 1-2 females were isolated according to the *Oamb-RFP* fluorescence for each experiment. ~10 intact follicles were pipetted into a well with 490 μL culture media. 15 z-positions (2 μm/each; 28 μm for each time point) were acquired at each time point at a frequency of one stack every 20 seconds. Both TexasRed (*Oamb-RFP*, to ensure follicles were in focus) and FITC (GCaMP) channels were collected. For each date of recording and each genotype, one video was recorded to measure the rate of signal decay throughout the duration of the imaging process. In this “control decay” video, 10 μL of culture media (CM) was added without any additional drug. For each experimental video, 10 μL of solution (containing CM+ octopamine (20 μM unless otherwise noted), CM+ ionomycin (5 μM), CM+ thapsigargin (10 μM)) was pipetted into the culture media in the imaging dish after frame ten, so the first ten frames were used as the “baseline”. For quantification, we utilized the Leica LAS-X software. We maximum-projected each time point, and a standardized region of interest (ROI; 45.38 um^2) was positioned in the middle/center of the egg chamber and the fluorescence intensity of the channel collecting GCaMP signal was analyzed. An egg chamber was not analyzed if: 1. It moved substantially, such that a ROI would not be covering one egg chamber throughout the duration of the video or 2. if the follicle ruptured. The following formula was used to achieve “adjusted ΔF/F0”, as plotted in the figures:

\[
F_{adj(n)} = F_{exp(n)} + \left[ \left( \frac{F_{con(0)} - F_{con(n)}}{F_{con(0)}} \right) F_{exp(0)} \right]
\]

Wherein “exp” is any experimental condition where a drug is added after frame 10 and “con” is the negative control video with just a vehicle added after frame 10. To measure area under the curve (Figure 4Q, Figure 5K, Figure S4D), Reimann sum was calculated in Microsoft.
Excel: the sum of each time point’s area (average adjusted ΔF/F0 of two neighboring time points * duration between those points). In Figure 4Q and Figure 5K and Figure S4D, every egg chamber’s AUC is plotted (circles) overlaying the average of all trials ± SD.

**in vivo**: Females were reared in 25 °C to 5 days old with 2 days supplemented with wet yeast paste. A drop of UV-curing glue (UV Knot Sense, Loon Outdoors, Boise, ID, USA) was placed on a glass microscope slide and a female was positioned on the droplet – dorsal-side toward the glue, with the majority of the thorax and abdomen in the glue droplet. Wings and legs were also positioned to be in the glue droplet. After correct positioning, the glue was cured with ~10 sec of UV light stimulation. The female was given ~5 minutes to wake from CO2 and adjust to the settings before image acquisition. Images were acquired at 0.5 Hz for 33 minutes.

**Immunostaining and microscopy**

DAPI staining was performed following a standard procedure as described previously. Ovaries were dissected, fixed in 4% EM-grade paraformaldehyde in PBST for 15 minutes, washed in PBST, then stained with DAPI (concentration?). Ovary pairs remained intact during staining and mounting. Images were acquired using a Leica Dmi8 and assembled using Photoshop software (Adobe Inc., Mountain View, CA) and Fiji (Schindelin et al., 2012).

**Statistical analysis**

Candidate calcium channels were chosen based upon a recent review (Chorna and Hasan, 2012). Expression levels obtained from a microarray database (Tootle et al., 2011) are listed for average stage-14 expression in arbitrary units. Expression levels obtained from a RNA-seq database are listed as (average stage-13 and stage-14) in RNA RPKM (Kronja et al., 2014). Oamb expression was plotted first as a reference. Prism 7 software was used for all statistical analysis. One-way ANOVA with Dunnett’s post-hoc analysis was performed for all egg-laying, mature follicle count, and ex vivo octopamine- or ionomycin- induced rupture assays. Chi-square
test was used for estimating ovulation time.

Acknowledgements

We are thankful for the generosity of many people for sharing fly lines: Drs. Allan Spradling, Dirk Bohmann, Mariana Wolfner, Benjamin White, and Andrea Page-McCaw, the Bloomington Drosophila Stock Center, and the Vienna Drosophila Stock Center. We are grateful for the LoTurco, Moiseff, and Nishiyama labs for sharing reagents and microscopes and Fred Murphy for assistance with UV-light curing glue for in vivo videos. We thank the anonymous reviewers for comments regarding the manuscript; and Elizabeth Knapp and Wei Li for discussions and technical support. The Leica SP8 confocal microscope is supported by a NIH award (S10OD016435) to Akiko Nishiyama. J.S. is supported by the University of Connecticut Start-up fund, NIH/National Institute of Child Health and Human Development Grant R01-HD086175, and Bill & Melinda Gates Foundation.
Supplemental Figure 4-1 Mature follicles after two-day egg laying experiment

Average number of mature follicles present in the ovary after a two-day egg laying experiment are plotted. FC1 Gal4 is plotted in black bars and FC2 Gal4 is plotted in grey bars. Number of females (egg-laying) is listed above each bar. *p < 0.05, **p < 0.01, ***p < 0.001.

Supplemental Figure 4-2 Overactive Gaq Causes precocious rupture
Expression and follicle-cell specific RFP (FC2>RFP; A-B) or hindsight (Hnt, C-D) in precociously-ruptured follicles. Quantification of egg laying for females expressing dominant-active Gaq mutation (E). Number of females is above each bar, ***p < 0.001.

Supplemental Figure 4-3 Calmodulin and CaMKII are required for follicle rupture

Egg laying capacity (A, E) and estimated ovulation time (B, F) in follicles with Cam-RNAi and CamKII-RNAi. Octopamine-stimulated (C, G) and ionomycin-stimulated (D, H) follicle rupture in follicles with Cam-RNAi and CamKII-RNAi. FC1 Gal4 is plotted in black bars and FC2 Gal4 is plotted in grey bars. Number of females (egg-laying) or number of replicates (ex vivo cultures) are listed above each bar. *p < 0.05, **p < 0.01, ***p < 0.001.
Supplemental Figure 4-4 Octopamine dose-response from GCaMP ex vivo imaging

One representative video was chosen wherein the response from each follicle in that video is plotted in grey and the average from that video is overlaid in color. The scale bar is 100% ΔF/F₀ (vertical) and 5 minutes (horizontal). At T= ~3 minutes, octopamine (“OA”) was added at 0.5 μM (A), 1 μM (B), or 5 μM (C). The area under the curve (“AUC”) average is plotted and the value for every follicle recorded is overlaid in colored circles (D). Number of egg chambers analyzed is above each bar.

Supplemental Figure 4-5 Thapsigargin dose-response from GCaMP ex vivo imaging

One representative video was chosen wherein the response from each follicle in that video is plotted in grey and the average from that video is overlaid in light orange (5 μM thapsigargin, A) or dark orange (20 μM thapsigargin, B). The scale bar is 100% ΔF/F₀ (vertical) and 5 minutes (horizontal). At T= ~3 minutes, thapsigargin (“Tg”) was added (arrow). The area under the curve (“AUC”) average is plotted and the value for every follicle recorded is overlaid in colored circles.
Supplemental Figure 4-6 Follicles with CaMKII-RNAi have a normal calcium response to GCaMP

One representative video was chosen wherein the response from each follicle in that video is plotted in grey and the average from that video is overlaid in color. The scale bar is 100% ΔF/F0 (vertical) and 5 minutes (horizontal). At T= ~3 minutes, octopamine (“OA”) was added. The area under the curve (“AUC”) average is plotted and the value for every follicle recorded is overlaid in colored circles (C). Number of egg chambers analyzed is above the bar.
Table 2. Egg Distribution with the reproductive tract and egg laying time statistics.

<table>
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<tr>
<th>Genotype</th>
<th>Eggs laid in 2 days</th>
<th>Egg distribution in 6hr</th>
<th>Egg laying time (min)</th>
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<tr>
<td></td>
<td>N</td>
<td>Uterus with egg (%)</td>
<td>Oviduct with egg (%)</td>
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<tr>
<td>UAS-dcr2/+; 44E10-Gal4/(Ore-R)</td>
<td>65</td>
<td>73.75 ± 1.24</td>
<td>52.22 ± 10.32</td>
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<tr>
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<td>80</td>
<td>42.28 ± 0.82 ***</td>
<td>32.14 ± 8.65</td>
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<tr>
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<td>40.00 ± 9.15</td>
</tr>
<tr>
<td>UAS-dcr2/+; 47A04-Gal4/(Ore-R)</td>
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<td>47.06 ± 9.69</td>
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<td>UAS-dcr2/Gaq<strong>RNAi</strong>; 47A04-Gal4/+</td>
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<td>24.07 ± 8.06</td>
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<tr>
<td>UAS-dcr2/Gaq<strong>RNAi</strong>; 47A04-Gal4/+</td>
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<td>16.07 ± 9.62</td>
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Eggs/female/day plotted as average in 22h ± 95% C.I.

Uterus with egg (%) plotted as Uterus% ± 95% C.I.

Ovulation time and uterus time are plotted as time ± 95% C.I.

* p<0.05

** p<0.01

*** p<0.001
CHAPTER 5: THE ZINC-FINGER HINDSIGHT REGULATES OVULATION COMPETENCY OF DROSOPHILA FOLLICLES

This chapter is published: Deady, L.D., Li, W., AND Sun, J. (2017). The Zinc-Finger Transcription Factor Hindsight Regulates Ovulation Competency of Drosophila Follicles. eLife 2017;6:E29887

Author contributions: Lylah D Deady, Formal analysis, Investigation, Writing—original draft, Writing—review and editing; Wei Li, Formal analysis, Investigation, Writing—review and editing; Jianjun Sun, Conceptualization, Formal analysis, Supervision, Funding acquisition, Project administration, Writing—review and editing

INTRODUCTION

Ovulation is a complex process of releasing fertilizable oocytes from mature follicles and is essential for animal reproduction (Espey and Richards, 2006). To ensure successful ovulation, a follicle must be developed to full maturity to be competent to receive an ovulatory stimulus and to activate proteolytic systems for follicle rupture. Several proteolytic systems have been found to regulate follicle rupture in vertebrates, including matrix metalloproteinase (Mmp), plasminogen activator/plasmin, and ADAMS-TS (Curry and Smith, 2006; Takahashi et al., 2013). In addition, a surge of luteinizing hormone (LH) serves as a master regulator to initiate the ovulation event and activates the EGF/EGFR-Ras-MAPK signaling pathway to propagate the ovulatory signal from outer granulosa cells to inner cumulus cells in the preovulatory follicles (Conti et al., 2012; Fan et al., 2009, 2011, 2012; Hsieh et al., 2007). However, molecular mechanisms coupling the Ras-MAPK pathway to the activation of proteolytic systems for follicle rupture are largely unknown.

Ovulation in Drosophila utilizes conserved molecular mechanisms and involves a follicle rupture process to release mature oocytes from the ovary. Drosophila have two ovaries, connected at their posterior end by bilateral oviducts (Figure 1). Each ovary contains ~16 ovarioles, where egg chambers are assembled in the germarium at the anterior and develop through 14 characteristic stages toward posterior end (Spradling, 1993). Each egg chamber contains one oocyte and 15 nurse cells surrounded by a layer of somatic follicle cells. In stage-
14 egg chambers (also named mature follicles), all nurse cells are degraded, leaving an oocyte surrounded by follicle cells; Matrix metalloproteinase 2 (Mmp2) is upregulated in posterior follicle cells (Figure 1; Deady et al., 2015). In addition, *Oamb* (*octopamine receptor in mushroom body*), encoding an α-adrenergic receptor-like G-protein coupled receptor for octopamine (OA), is also upregulated in all follicle cells of stage-14 egg chambers (Lee et al., 2003; Deady and Sun, 2015). OA, released from terminal nerves that innervate ovaries, activates Oamb receptor in stage-14 follicle cells, which induces calcium rise and activates Mmp2 (Deady and Sun, 2015; Heifetz et al., 2014; Middleton et al., 2006; Monastirioti, 2003). Mmp2 enzymatic activity leads to degradation of posterior follicle cells and release of the encapsulated oocyte (called follicle rupture; Figure 1; Deady et al., 2015). The rest of the follicle cells remain at the end of the ovariole to form a corpus luteum (Deady et al., 2015). Local adrenergic signaling has also been suggested to regulate mammalian ovulation but no molecular mechanisms have been illustrated (Kannisto et al., 1985; Schmidt et al., 1985). In parallel to progesterone signaling in mammalian ovulation, ecdysteroid signaling is also activated in stage-14 follicle cells and is essential for *Drosophila* ovulation; ecdysteroid signaling modulates OA/Oamb-induced Mmp2 activation, but does not affect Oamb expression nor Mmp2 expression (Knapp and Sun, 2017). Thus, it is currently unknown what induces Mmp2 and Oamb expression in stage-14 follicle cells and how these follicles become fully competent for ovulation.
Figure 5.1 An illustration of *Drosophila* ovulation process

The female reproductive system, consisting of two ovaries, oviduct, uterus, seminal receptacle, and a pair of spermathecae and parovaria, was depicted in the cartoon. Two representative ovarioles with different staged egg chambers were highlighted in the right ovary. Oocytes and nurse cells are in yellow. Mmp2 expression is shown in green and Mmp2 activity is shown in red.

The zinc-finger transcription factor Hindsight (Hnt; encoded by gene *pebbled*) contains 14 C2H2 zinc-finger domains and is homologous to mammalian Ras-responsive element-binding protein 1 (RREB-1). Both Hnt and RREB-1 bind to similar DNA sequences, and human RREB-1 can functionally replace Hnt in attenuating expression of *nervy* and *hnt* itself in *Drosophila* salivary gland (Ming et al., 2013). RREB-1 functions downstream of the Ras-MAPK pathway to either suppress or promote Ras target genes in multiple tissues including colon, thyroid, and pancreatic cancers (Kent et al., 2010, 2013; Mukhopadhyay et al., 2007; Thiagalingam et al., 1996; Zhang et al., 2003). Hnt is expressed in a variety of tissues in development and plays multiple developmental roles including control of embryonic germ band retraction (Yip et al., 1997; Reed et al., 2001), regulation of retinal cell fate and morphogenesis.
(Pickup et al., 2002; Wilk et al., 2004; Pickup et al., 2009; Oliva and Sierralta, 2010; Oliva et al., 2015), maintenance of tracheal epithelial integrity (Wilk et al., 2000, 2004), and differentiation of spermathecae and intestinal stem cells (Sun and Spradling, 2013; Baechler et al., 2015). Hnt is also expressed in follicle cells of stage 7-10A egg chambers, where it functions as a downstream target of Notch signaling to suppress Hedgehog signaling and to induce the mitotic/endocycle transition (Sun and Deng, 2007). Hnt continues its expression in anterior follicle cells throughout late oogenesis. In contrast, Hnt expression in main body follicle cells is downregulated from stage 10B to stage 13 and re-upregulated in stage-14 (Deady et al., 2015), where its role is unknown. Moreover, few downstream targets of Hnt have been identified and its relationship to Ras signaling is also unknown.

Here, we characterized the dynamic expression of Hnt in stage-14 follicle cells. By using molecular and genetic tools, we demonstrated that Hnt expression in stage-14 follicle cells is essential for follicle rupture partly by upregulation of Oamb and Mmp2 expression in these follicle cells. Thus, Hnt functions as an essential transcription factor to prime follicles to be competent for follicle rupture/ovulation. In addition, Hnt's role in follicle rupture can be replaced by human RREB-1. Our data, along with the involvement of Ras-MAPK signaling in mammalian ovulation, lead us to propose that Hnt/RREB-1 has a conserved role in regulating follicle rupture/ovulation downstream of Ras-MAPK signaling pathway.

RESULTS

Dynamic expression of Hindsight in stage-14 follicle cells

Hnt is not expressed in stage-13 follicle cells except those at the anterior region; however, it is upregulated in all stage-14 follicle cells and the corpus luteum (Deady et al., 2015). Upon closer examination, we found three distinct patterns of Hnt expression throughout stage-14 egg chambers: I) high Hnt expression in anterior and posterior but low/no Hnt in the middle follicle.
cells ("A/P-Hnt" egg chambers; Figure 2A and F, Figure S1A-B); II) high Hnt expression in all follicle cells ("high-Hnt" egg chambers; Figure 2B and G); III) low Hnt expression in all follicle cells ("low-Hnt" egg chambers; Figure 2C and H; also see Figure S1C-D). To determine the developmental sequence of the aforementioned three types of stage-14 egg chambers, we analyzed the expression patterns of Hnt against the expression of a stage-14 follicle-cell Gal4 driver (44E10-Gal4; renamed as FC1 for simplicity) (Deady and Sun, 2015) and the number of nurse cell nuclei in these egg chambers. 86% of A/P-Hnt egg chambers had medium-level GFP expression driven by FC1, while more than 73% of high-Hnt and 80% of low-Hnt egg chambers had high-level GFP expression (Figure 2A-D). This indicates that A/P-Hnt egg chambers are the youngest, which is consistent with the observation that A/P-Hnt egg chambers typically have more residual nurse-cell nuclei than the other two types of egg chambers (Figure 2E). In addition, high-Hnt egg chambers still contained one or two nurse-cell nuclei, while low-Hnt egg chambers typically did not contain nurse-cell nuclei and were skinner and dehydrated (Figure 2E and Figure S1D). Because nurse-cell nuclei are progressively degraded starting around stage-12 by a non-cell-autonomous mechanism to generate fully matured egg chambers, which have no nurse cell nuclei and are dehydrated (Drummond-Barbosa and Spradling, 2004; Timmons et al., 2016), the above analysis demonstrates that high-Hnt egg chambers are at the intermediate stage, while low-Hnt egg chambers are the most mature egg chambers.

This conclusion was further supported by additional analysis using a late stage-14 follicle-cell Gal4 driver (47A04-Gal4; renamed as FC2 for simplicity) (Deady and Sun, 2015). Consistent with the previous result, both A/P- and high-Hnt egg chambers had no or minimal GFP expression driven by FC2, while low-Hnt egg chambers had highest GFP expression and fewest nurse-cell nuclei (Figure 2F-J). Altogether, these analyses demonstrate that Hnt is first upregulated in posterior follicle cells, filled in across the entire egg chamber, and then overall downregulated in follicle cells of fully matured egg chambers (Figure 2K). Therefore, we propose to categorize
stage-14 egg chambers into three distinct stages and rename A/P-, high-, and low-Hnt egg chambers as stage-14A, stage-14B, and stage-14C egg chambers, respectively (Figure 2K).

Figure 2

Figure 5.2 Hindsight expression in stage-14 egg chambers

(A–C) Hnt expression (red in A–C and white in A’–C’) in A/P-Hnt (A), high-Hnt (B), and low-Hnt (C) egg chambers. FC1 expression (FC1-Gal4 driving UAS-eGFP, FC1 > GFP) is shown in green (A–C) and white in (A’–C’). Nuclei are shown by DAPI in blue (A–C). (D–E) Quantification of FC1 expression (D) and residual nurse cell nuclei (E) in A/P-Hnt, high-Hnt, and low-Hnt egg chambers.
The number of stage-14 egg chambers analyzed is noted above each bar. (F–H) Hnt expression (red in F–H and white in F'–H') in A/P-Hnt (F), high-Hnt (G), and low-Hnt (H) egg chambers. FC2 expression (FC2 > GFP) is shown in green (F–H) and white (F'–H'). Nuclei are shown in blue (F–H). (I–J) Quantification of FC2 expression (I) and residual nurse cell nuclei (J) in A/P-Hnt, high-Hnt, and low-Hnt egg chambers. (K) A schematic cartoon shows the temporal pattern of Hnt, FC1 and FC2 expression in stage-14 egg chambers. FC1-related graphs are colored green and FC2-related graphs are colored blue.

**Hindsight in stage-14 follicle cells is required for normal ovulation**

The dynamic Hnt expression in stage-14 follicle cells prompted us to investigate its function in follicle maturation and ovulation. To bypass the early requirement of Hnt in follicle cell differentiation (Sun and Deng, 2007), we used RNA interference (RNAi) to deplete Hnt expression specifically in stage-14 follicle cells with FC1 or FC2 Gal4 driving UAS-hnt\textsuperscript{RNAi} expression. While FC1 started to be expressed in stage-14A follicle cells, it was weak to deplete Hnt expression in stage-14A follicle cells (Figure S2A-D) but became progressively more efficient in older follicle cells with two independent hnt\textsuperscript{RNAi} lines (Figure 3A-D and Figure S2E-H); more than 80% of stage-14C egg chambers had no detectable Hnt expression in their follicle cells. In contrast, FC2 started to be expressed in stage-14B follicle cells and most effectively depleted Hnt expression in stage-14C follicle cells except with hnt\textsuperscript{RNAi2}, which only showed strong reduction in ~43% of egg chambers (Figure 3E-H and Figure S2I-P).

Females with RNAi-mediated hnt depletion in stage-14 follicle cells (named hnt\textsuperscript{RNAi} females for simplicity) were then assayed for egg-laying ability. hnt\textsuperscript{RNAi} females laid significantly fewer eggs than control females after mating (Figure 3I). This phenotype was manifested by using both stage-14 follicle-cell Gal4 drivers and with two independent hnt\textsuperscript{RNAi} lines, which demonstrates that Hnt expression in stage-14 follicle cells was essential for normal egg laying. The decrease in egg-laying number was not caused by an oogenesis defect, as plenty of stage-14 egg chambers were observed before and after egg-laying experiments (Figure 3J and Figure S3). The egg-laying process consists of ovulation (the release of egg from the ovary into the oviduct), egg
transportation through the oviduct, and oviposition (the release of egg in the uterus to the outside environment). To determine which step in the egg-laying process was affected in \textit{hnt^{RNAi}} females, we utilized our previously developed method to estimate the average time required for each step in the egg-laying process (Deady and Sun, 2015; Knapp and Sun, 2017; Sun and Spradling, 2013). Consistent with our previous data, control females spent 12 - 14 minutes to ovulate an egg, less than a minute to transport egg through the oviduct, and eight - 10 minutes to hold an egg in the uterus and oviposit (Figure 3K-L and Table 1). In contrast, \textit{hnt^{RNAi}} females spent more than 25 minutes to ovulate an egg, which was significantly longer than the control females (Figure 3K-L and Table 1). These data demonstrate that Hnt in stage-14 follicle cells is required for normal ovulation.
Figure 3. Hindsight expression in stage-14 follicle cells is essential for normal ovulation.

(A–D) Hnt expression (green) in control (A) and hnt-RNAi (B–C) stage-14C egg chambers with FC1. FC1 expression (FC1 > RFP) is shown in red. Inserts are high magnification of Hnt expression in squared areas. The quantification of Hnt expression (categorized as high-Hnt, low-Hnt, A/P-Hnt, and None-Hnt) in stage-14C egg chambers is shown in (D). The number of stage-14C egg chambers (selected according to no nurse-cell nuclei/high FC1 expression) analyzed is noted above each bar. (E–H) Hnt expression (green) in control (E) and hnt-RNAi (F–G) stage-14C egg chambers with FC2. FC2 expression (FC2 > RFP) is shown in red. Inserts are high magnification of Hnt expression in squared areas. The quantification of Hnt expression in stage-14C egg chambers is shown in (H). The stage-14C egg chambers are selected according to no nurse-cell nuclei/high FC2 expression. (I–J) The quantification of egg laying (I) and mature egg chambers in each female’s ovaries after egg laying (J) in control or hnt-RNAi females with FC1- (green bars) or FC2-Gal4 (blue bars). The number of females is noted above each bar. (K–L) The egg-laying time in control or hnt-RNAi females with FC1 (K) or FC2 (L). Also see Table 1. *p<0.05, **p<0.01, ***p<0.001.
Hindsight in stage-14 follicle cells is necessary for OA-induced follicle rupture

Ovulation consists of a breakdown of posterior follicle cells and a subsequent rupture of oocyte into the lateral oviduct (Figure 1), which is induced by octopaminergic signaling and can be recapitulated in an ex vivo culture system (Deady and Sun, 2015). The requirement of Hnt for normal ovulation led us to hypothesize that Hnt is required for OA-induced follicle rupture. Consistent with this idea, about 45% stage-14 egg chambers isolated according to FC1 expression from control females ruptured in response to OA stimulation, whereas fewer than 10% of egg chambers from hntRNAi females ruptured in response to OA stimulation (Figure 4A-D). In addition, more than 85% stage-14 egg chambers isolated according to FC2 expression from control females ruptured in response to OA stimulation (Figure 4E and 4H), consistent with the fact that FC2 is expressed in more mature egg chambers than FC1 (Figure 2). In contrast, egg chambers isolated from hntRNAi or hntRNAi females with FC2 ruptured at the rate of 10% and 33%, respectively (Figure 4F-H). Consistent with this result, follicles isolated from hnt transheterozygous mutant females also showed significant reduction in OA-induced follicle rupture in comparison to control follicles (Figure S4). These results demonstrate that Hnt is required in stage-14 follicle cells for follicle rupture.
Hindsight is essential for Mmp2 activity in posterior follicle cells of stage-14 egg chambers

Mmp2 is essential for follicle rupture, and its enzymatic activity is activated by OA stimulation (Deady and Sun, 2015). To determine whether Hnt regulates follicle rupture by influencing Mmp2 activity, we assayed Mmp2 enzymatic activity in egg chambers from control and hnt-RNAi females after OA stimulation ex vivo. After a three-hour incubation with OA, ~60% of control egg chambers isolated according to FC1 expression had posterior gelatinase activity (Figure 5A and D), whereas only ~25% of hnt-RNAi egg chambers had posterior gelatinase activity (Figure 5B-D). In addition, about 90% of control egg chambers isolated according to FC2 had posterior gelatinase activity, in contrast to 25% and 47% of hnt-RNAi and hnt-RNAi2 egg chambers, respectively (Figure 5E-H). The proportion of follicles with posterior gelatinase activity was

Figure 5.4 Hindsight is required for OA-induced follicle rupture

(A–D) Representative images show control (A) and hnt-RNAi (B–C) egg chambers with FC1 after three-hour culture with 20 μM OA. Quantification of OA-induced follicle rupture is shown in (D). (E–H) Representative images show control (E) and hnt-RNAi (F–G) egg chambers with FC2 after three-hour culture with 20 μM OA. Quantification is shown in (H). Egg chambers were isolated according to FC1 > RFP (red in A–C) or FC2 > RFP (red in E–G) expression. Bright-field images of the egg chambers are shown in blue, and ruptured egg chambers are marked by white arrowheads. The number of egg chambers is listed above each bar. ***p<0.001.
correlated to the proportion of follicles that ruptured, and both were significantly decreased in hnt\textsuperscript{RNAi} egg chambers, which strongly supports that Hnt controls follicle rupture by regulating Mmp2 activity.

Figure 5

(A–C) Representative images show OA-induced Mmp2 activity (green) in control (A) and hnt-RNAi (B–C) egg chambers with FC1 after three-hour culture with OA. Egg chambers with posterior Mmp2 activity are marked by arrowheads. Quantification is shown in (D). (E–H) Representative
images show OA-induced Mmp2 activity (green) in control (E) and hnt-RNAi (F–G) egg chambers with FC2 after three-hour culture with OA. The quantification is shown in (H). (I) A diagram shows the three categories of basement-membrane (BM) configurations (according to Vkg::GFP expression) of follicle cells in isolated stage-14 egg chambers. When a line connecting the posterior-most Vkg edges bisects the oocyte, it is defined as an open-BM configuration, whereas when the line does not bisect the oocyte, it is defined as a broken-BM configuration. The intact-BM configuration is defined as intact, continuous Vkg::GFP throughout the posterior of the egg chamber. (J–J’) A control egg chamber identified according to FC2 > RFP expression shows the open-BM configuration. (K–K’) An egg chamber with overexpression of Timp shows the intact-BM configuration. (L–L’) An Mmp2-RNAi egg chamber shows the broken-BM configuration. (M–N) hnt-RNAi egg chambers show intact-BM (M–M’) and broken-BM (N–N’) configurations. (O) Quantification of BM configurations of FC2 expressing egg chambers with respective genotypes. The number of egg chambers analyzed is noted above each bar. ***p<0.001.

To further support this notion and to avoid the possibility that the above observed phenomenon is an artifact of ex vivo culture, we determined whether Hnt indeed regulates Mmp2 activity in vivo. One of the known substrates of Mmp2 is the basement-membrane (BM) protein collagen IV, encoded by viking (vkg), during imaginal disc morphogenesis and fatbody dissociation at pupal development (Srivastava et al., 2007; Jia et al., 2014). Vkg is detected in the basement membrane of follicle cells throughout oogenesis and could be a substrate of follicular Mmp2 as well. We found that 70% of FC2-expressing egg chambers had lost follicular Vkg protein in a large posterior area (open-BM configuration), 26% had lost Vkg protein in a small posterior area (broken-BM configuration), and 4% had intact Vkg protein (intact-BM configuration) at their posterior end (Figure 5I–J and O). When tissue inhibitor of matrix metalloproteinase (Timp, encoding an inhibitor of Mmp enzymatic activity) was overexpressed in stage-14 follicle cells using FC2, the BM configuration was dramatically shifted toward intact-BM configuration (Figure 5K and O), indicating that Mmp activity is responsible for the degradation of Vkg at the posterior end of stage-14 egg chambers. In addition, RNAi-mediated Mmp2 depletion in stage-14 follicle cells showed a similar trend as overexpression of Timp, although less effectively (Figure 5L and O), demonstrating that Mmp2 is, at least partially, responsible for the Vkg degradation. Furthermore, hnt depletion in stage-14 follicle cells also shifted BM configuration toward broken- and intact-BM configuration as Mmp2 depletion (Figure 5M–O).
Altogether, these data demonstrate that Hnt regulates Mmp2 activity, which is responsible for Vkg degradation at the posterior end of stage-14 egg chambers during ovulation.

**Hindsight is required for Mmp2 expression in posterior follicle cells at stage 14**

OA binds to Oamb receptor in stage-14 follicle cells, which leads to a rise of intracellular Ca\(^{2+}\) concentration and subsequent activation of Mmp2 (Deady and Sun, 2015). To elucidate the mechanism of Hnt in regulating Mmp2 activity, we sought to determine whether Hnt interferes with OA/Oamb-Ca\(^{2+}\)-Mmp2 pathway upstream and/or downstream of Ca\(^{2+}\) rise. In comparison to OA, Ca\(^{2+}\) ionophore ionomycin was sufficient to induce more than 95% control egg chambers to rupture at the end of a three-hour culture, regardless whether egg chambers were isolated according to FC1 or FC2 expression (Figure 6A, D, E, and H). In contrast, ionomycin was still not sufficient to induce follicle rupture in hnt\(^{RNAi}\) egg chambers (except those with FC2 driving hnt\(^{RNAi2}\) expression; Figure 6B-D and F-H), despite that it was able to induce Ca\(^{2+}\) rise in follicle cells (Figure S5A-D, Videos 1-3). The incompetency of hnt\(^{RNAi}\) egg chambers to ionomycin stimulation indicates that Hnt regulates components downstream of Ca\(^{2+}\) rise in the OA/Oamb-Ca\(^{2+}\)-Mmp2 pathway; the almost normal response to ionomycin but defective response to OA in hnt\(^{RNAi2}\) egg chambers with FC2 indicates that Hnt also regulates components upstream of Ca\(^{2+}\) rise in the OA/Oamb-Ca\(^{2+}\)-Mmp2 pathway. Consistent with this idea, OA was not sufficient to induce Ca\(^{2+}\) rise in hnt\(^{RNAi}\) egg chambers with FC2 (Figure S5E-H, Videos 4-6). Since Hnt is first upregulated in posterior follicle cells (Figure 2), where Mmp2 is expressed (Deady et al., 2015) and then swept across the entire follicle cells (Figure 2), where Oamb is expressed (Lee et al., 2003; Deady and Sun, 2015), we hypothesize that Hnt regulates both Mmp2 and Oamb expression in stage-14 follicle cells.
Figure 5.6 Hindsight regulates Mmp2 expression in stage-14 follicle cells

(A–H) Response of egg chambers isolated according to FC1 (A–D) or FC2 (E–H) to ionomycin-induced rupture in three hours. (A–C and E–G) Representative images show control (A and E) and hnt-RNAi (B–C and F–G) egg chambers after the culture. Bright field images of the egg chambers are shown in blue, and white arrowheads mark ruptured egg chambers. Quantification of rupture response to OA or to ionomycin is shown in (D and H). (I–K) Representative images show Mmp2::GFP expression (green in I–K and white in I’–K’) in control (I–I’) or hnt-RNAi (J–K’) egg chambers with FC2 Gal4 (Red). Nuclei are labeled with DAPI and shown in blue (I–K). Arrowheads point to posterior follicle cells, and oocytes are outlined in cyan (I’–K’). (L–M) Quantification of Mmp2::GFP expression in control and hnt-RNAi egg chambers using FC1-Gal4
(L) or FC2-Gal4 (M). (N–O) Quantification of Mmp2 mRNA levels in hnt-RNAi egg chambers with FC1-Gal4 (N) or FC2-Gal4 (O). *p<0.05, **p<0.01, ***p<0.001.

To investigate the role of Hnt in Mmp2 expression, we examined Mmp2 expression using a Mmp2::GFP fusion gene in the endogenous locus. Consistent with our previous report, Mmp2::GFP was detected in posterior follicle cells of stage-14 egg chambers, most prominently in stage14B and 14C (Figure 6I and Figure S6A). Mmp2::GFP formed a gradient that peaked at the posterior tip and gradually decreased toward the anterior. In contrast, there was marked reduction of Mmp2::GFP intensity in hntRNAi egg chambers (Figure 6J-K and Figure S6B-C). More than 80% of FC1- or FC2-expressing control egg chambers had moderate or high-level of Mmp2::GFP expression in their posterior follicle cells, while fewer than 30% of hntRNAi egg chambers (32% in the case of FC2 driving hntRNAi2) had moderate or high-level of Mmp2::GFP expression (Figure 6L-M). Due to technical challenges, we were unable to quantify Mmp2 protein level directly using western blotting. However, we speculated that Hnt might regulate Mmp2 transcription. Therefore, we used real-time RT-PCR to quantify Mmp2 mRNA level in control and hntRNAi egg chambers. Consistent with this hypothesis, Mmp2 mRNA levels were significantly decreased in hntRNAi egg chambers in comparison to the control (Figure 6N-O). Altogether, these data demonstrate that Hnt regulates Mmp2 expression at the transcriptional level.

**Hindsight is required for Oamb expression in stage-14 follicle cells**

We noticed that hntRNAi2 egg chambers with FC2 Gal4 had slightly weaker reduction of Mmp2 mRNA and protein expression (Figure 6M and O) and responded normally to ionomycin stimulation (Figure 6H) but were defective in OA-induced Ca^{2+} rise, Mmp2 activation, and follicle rupture (Figure 4H, 5H, and Figure S5E-H). This suggests that components upstream of Ca^{2+} rise, for example Oamb, are defective in these egg chambers. Consistent with this hypothesis, Oamb mRNA was reduced two or more folds in hntRNAi egg chambers regardless the Gal4 drivers or hntRNAi lines (Figure 7A-B). Therefore, Hnt is also required for Oamb expression in stage-14 follicle
cells.

Figure 5.7 Hindsight regulates Oamb expression in stage-14 follicle cells

(A–B) Quantification of Oamb mRNA levels in hnt-RNAi egg chambers with FC1-Gal4 (A) or FC2-Gal4 (B). (C–D) Quantification of egg chambers in response to OA-induced follicle rupture. hnt-RNAi and/or Oamb overexpression is driven by FC1-Gal4 (C) or FC2-Gal4 (D). (E–L) Representative images of the quantification in (C–D). FC1 >RFP (E–H) or FC2 >RFP (I–L) is shown in red, bright-field images of the egg chambers are shown in blue, and ruptured egg chambers are marked by white arrowheads. *p<0.05, **p<0.01, ***p<0.001.
Next, we aimed to rescue the rupture defect of $hnt^{RNAi}$ egg chambers by overexpression of $Oamb$. $Oamb$ overexpression was not able to restore the competency to OA-induced rupture in $hnt^{RNAi}$ egg chambers with FC1 or $hnt^{RNAi}$ egg chambers with FC2, but it was able to do so in $hnt^{RNAi2}$ egg chambers with FC2 (Figure 7C-D), consistent with the ionomycin experiment (Figure 6D and H). These data suggest that the major defect in $hnt^{RNAi2}$ egg chambers with FC2 is the disruption of $Oamb$ expression, while $hnt^{RNAi1}$ egg chambers with FC2 or $hnt^{RNAi}$ egg chambers with FC1 have more defects than $Oamb$ alone, such as Mmp2 expression. The combination of later FC2 and weaker $hnt^{RNAi2}$ may explain why only $Oamb$ is majorly affected in this genetic manipulation. In addition, we noticed that egg chambers with $Oamb$ overexpression alone initiated rupture before being able to perform the ex vivo culture (i.e. few intact egg chambers could be isolated). This is likely due to its high $Oamb$ expression, which leads to high sensitivity to very low amount of endogenous OA released during egg chamber isolation. Nevertheless, all these data support the notion that Hnt transcriptionally upregulates Mmp2 expression in posterior follicle cells and then $Oamb$ expression in all follicle cells to make stage-14 egg chambers to be competent to respond to OA-induced follicle rupture.

**Human RREB-1 can replace Hindsight’s role in regulating follicle’s competency to ovulation**

To address whether the role of Hnt in stage-14 follicle cells can be replaced by its human homolog RREB-1, we first aimed to rescue the defects of $hnt^{RNAi}$ egg chambers with $hnt$ overexpression using $hnt^{EP55}$ (see materials and methods). To our surprise, overexpression of $hnt$ in $hnt^{RNAi}$ egg chambers did not rescue their defect in OA-induced follicle rupture (Figure S7A and C). In addition, these females laid similar numbers of eggs as $hnt^{RNAi}$ females (Figure S7 and D). Surprisingly, Hnt protein was still depleted despite using FC1 or FC2 Gal4 driving $hnt^{EP55}$ expression (Figure S7E-L), indicating that $hnt^{RNAi}$ is sufficient to disrupt overexpressed $hnt$ mRNA. This was further validated in a flip-out Gal4 system, in which Hnt protein was greatly reduced in
cells with both hntRNAi and hntEP55 (Figure S7M-P). Despite the failure to rescue ovulation in hnt-depleted females, it is interesting to note that overexpression of hnt alone with FC1 or FC2 Gal4 driver enhanced and suppressed OA-induced follicle rupture, respectively (Figure S7A and C), suggesting that dynamic upregulation and downregulation of Hnt in stage-14 follicle cells may be required for normal function of these cells.
Figure 5.8 Human RREB-1 can replace Hindsight’s role in regulating follicle’s competency to ovulate.

(A) The quantification of egg-laying capacity of females with FC1 driving hnt-RNAi and/or RREB-1 overexpression. (B–F) The quantification of OA-induced follicle rupture (B) in egg chambers with hnt-RNAi and/or RREB-1 overexpression using FC1 Gal4. Representative images are shown in (C–F). FC1 >RFP is shown in red, bright-field images of egg chambers are shown in blue, and white arrowheads mark ruptured follicles. (G–H) Quantification of Oamb (G) and Mmp2 (H) mRNA level in egg chambers with FC1 Gal4 driving hnt-RNAi and/or RREB-1 overexpression. *p<0.05, **p<0.01, ***p<0.001.
Next, a functional RREB-1::GFP fusion gene was overexpressed in hntRNAi females with FC1 to see whether RREB-1 could rescue the ovulation defect of hntRNAi females. RREB-1 is successfully overexpressed in hntRNAi egg chambers, and overexpression of RREB-1 did not affect Hnt expression in control nor hntRNAi egg chambers (Figure S8A-F). hntRNAi2/RREB-1::GFP females showed significant increase of egg-laying number in comparison to hntRNAi2 females, indicating a rescue of ovulation defect (Figure 8A). This is supported by the result that hntRNAi2/RREB-1::GFP females spent 13 minutes, in comparison to 27 minutes in hntRNAi2 females, to ovulate an egg, close to that in control females (Table 1). In contrast, females with hntRNAi1/RREB-1::GFP laid significantly fewer eggs than females with hntRNAi1 alone (Figure 8A) and spent even longer time to ovulate an egg (Table 1). In addition, we noticed that these females frequently have eggs in the oviduct (Table 1), which may be caused by more frequent and uncoordinated follicle rupture leading to egg jamming in the oviduct. The persistence of egg in the oviduct may feedback to the ovary to inhibit further ovulation in vivo.

To more directly investigate the role of RREB-1 in ovulation, we isolated stage-14 egg chambers and performed OA-induced follicle rupture ex vivo. Excitingly, RREB-1::GFP overexpression was sufficient to rescue the rupture defect of hntRNAi egg chambers (Figure 8B-F), whereas overexpression of UAS-GFP was insufficient (Figure S9). In addition, overexpression of RREB-1 alone led to increased OA-induced follicle rupture, similar to overexpression of hnt with FC1 (Figure 8B and Figure S7A). Consistent with the rescue of follicle rupture, both Mmp2 and Oamb mRNA expression was rescued to normal or even higher level by overexpression of RREB-1 (Figure 8G-H). Therefore, RREB-1 can replace Hnt’s role in upregulating Mmp2 and Oamb expression in follicle cells. Altogether, our data demonstrate that zinc-finger transcription factor Hnt/RREB-1 may play conserved roles in promoting follicle maturation and ovulation competency.
Hnt expression is shown in red with different intensity indicating different expression level. Mmp2 expression is shown in green and Oamb expression is shown in blue. OA stands for octopamine.

**DISCUSSION**

**Hindsight regulates ovulation competency in stage-14 egg chambers**

Work in this study demonstrated for the first time that Hnt has a dynamic expression pattern in stage-14 follicle cells and is a key factor for the final maturation of stage-14 egg chambers (Figure 9). Oocyte maturation has been well studied in *Drosophila* and other
species (Eichhorn et al., 2016; Kronja et al., 2014; Stetina and Orr-Weaver, 2011); however, the maturation of follicle cells surrounding the oocyte in the stage-14 egg chamber is poorly defined at the molecular level (Duhart et al., 2017; Klusza and Deng, 2011; Spradling, 1993). According to Hnt expression in stage-14 egg chambers, we define the stage-14 egg chambers into three sub stages. Hnt is first upregulated in posterior follicle cells of stage-14A egg chambers, which is likely corresponding to Hnt’s role in upregulating Mmp2 expression in these follicle cells (Figure 9). Then Hnt is upregulated in all main-body follicle cells of stage-14B egg chambers, which is likely corresponding to Hnt’s role in upregulating Oamb expression (Figure 9). The sequential upregulation of Mmp2 and Oamb is fully consistent with the fact that FC1-expressing egg chambers, in comparison to FC2-expressing egg chambers, are less efficient for OA-induced follicle rupture, but fully competent to respond to ionomycin-induced follicle rupture (Figure 4D, 4H, 6D, 6H). The orchestrated upregulation of Mmp2 and Oamb, and possibly other components in the OA/Oamb-Ca^{2+}-Mmp2 pathway, by Hnt makes the final stage-14C egg chambers fully competent for ovulation. Components in the ecdysteroid signaling pathway, including the enzyme Shd for steroid production and Ecdysone receptor (EcR), also changes its expression pattern from stage-13 to stage-14 (Knapp and Sun, 2017). It is unknown whether Hnt is also responsible for such changes; however, it is unlikely that ecdysteroid signaling upregulates Hnt in stage-14 follicle cells, because ecdysteroid signaling does not affect Mmp2 and Oamb expression (Knapp and Sun, 2017). Hnt is upregulated in follicle cells from stage 7 to stage 10A, which depends on Notch signaling (Sun and Deng, 2007); however, Notch signaling is not active in stage-14 follicle cells and is unlikely to upregulate Hnt at this stage. Thus, the developmental signal for Hnt upregulation in stage 14 and the transition from stage 13 to stage 14 is still unknown.

**Hindsight and its role in extracellular matrix homeostasis and epithelial integrity**

Mmp2, along with Mmp1, are the only genes in the fly genome encoding matrix metalloproteinase and are crucial for extracellular matrix homeostasis during normal
development, wound repair, and cancer metastasis (Page-McCaw, 2008; Stevens and Page-McCaw, 2012). Unlike Mmp1, whose expression is tightly regulated by Jun-related kinase (JNK) signaling (Uhlrirova and Bohmann, 2006), regulation of Mmp2 expression is largely unknown. Our work clearly defines the role of Hnt in regulating Mmp2 expression and basement membrane remodeling during ovulation. Hnt directly binds to two adjacent Hnt-binding sequences in the regulatory region of hnt and nervy genes and attenuates their expression (Ming et al., 2013). Such Hnt-binding motifs are not found in the gene region of Mmp2 and Oamb. Thus, Hnt may indirectly regulate Mmp2 expression in posterior follicle cells. In addition, other transcriptional regulators must exist to coordinate with Hnt to restrict Mmp2 expression to posterior follicle cells.

Hnt’s role in regulating Mmp2 expression and extracellular matrix homeostasis may not be restricted to posterior follicle cells. It has been shown that Hnt has a general role in regulating epithelial integrity in multiple organ systems and developmental stages. During retinal morphogenesis, hnt mutant photoreceptor cells frequently delaminate from retinal epithelium and are unable to maintain their integrity (Pickup et al., 2002). In the tracheal system, hnt mutant tracheal epithelium disintegrate to form sacs and vesicles from collapsed dorsal trunk and branches (Wilk et al., 2000). During oogenesis, Hnt is essential for proper cell adhesion and collective cell migration in stage-9 egg chambers. Ectopic expression of Hnt in the cluster of border cells leads to dissociation of the border-cell cluster (Melani et al., 2008). In addition, genetic modifier screens identify basement-membrane components Vkg and Laminin as Hnt’s genetic interactors (Wilk et al., 2004). All of these studies suggest that Hnt plays general roles in regulating epithelial integrity and extracellular matrix homeostasis in multiple organ systems. It will be interesting to see whether the regulation of Mmp2 by Hnt also occurs in other Hnt-expressing or Mmp2-expressing tissues/organs.

**Hindsight and human RREB-1 are functionally conserved in ovulation**

*Drosophila* Hnt and mammalian RREB-1 are functionally conserved in many aspects. Both
Drosophila Hnt and mammalian RREB-1 are required for proper cell migration (Melani et al., 2008). Human RREB-1 binds to similar DNA sequences in Drosophila salivary gland polytene chromosomes as Hnt and rescues the germ band retraction phenotype in hnt mutant embryos (Ming et al., 2013). In addition, our current work shows that human RREB-1 is able to rescue Oamb and Mmp2 expression in stage-14 follicle cells and OA-induced follicle rupture/ovulation phenotype in hntRNAi females (Figure 8). The role of RREB-1 in mammalian ovulation has not been studied so far, however, RREB-1 is detected in granulosa cells in mouse ovaries by microarray analysis (Fan et al., 2009). In addition, mammalian RREB-1 functions downstream of the Ras-MAPK signaling pathway in multiple occasions (Kent et al., 2010, 2013; Mukhopadhyay et al., 2007; Thiagalingam et al., 1996; Zhang et al., 2003), and the Ras-MAPK signaling pathway is involved in mammalian ovulation (Fan et al., 2009). It is possible that RREB-1 may function in granulosa cells to regulate Mmp expression and ovulation downstream of Ras-MAPK pathway in mammals.

**MATERIALS & METHODS**

**Drosophila genetics**

Flies were reared on standard cornmeal and molasses food at 25°C, and all RNAi-mediated depletion experiments were performed at 29°C with UAS-dcr2. Two stage-14 follicle-cell specific Gal4 drivers from the Janelia Gal4 (Pfeiffer et al., 2008) collection were used in this study: R44E10-Gal4 (FC1) and R47A04-Gal4 (FC2). The following RNAi lines were used: UAS-hntRNAi1 (V3788) and UAS-hntRNAi2 (V101325) from the Vienna Drosophila Resource Center; and UAS-Mmp2RNAi (Uhlirova and Bohmann, 2006a). UAS-Oamb.K3 (Lee et al., 2009), UAS-Timp (Page-McCaw et al., 2003), hntEP55 (a P-element insertion line containing UAS sequence in the promoter region of hnt; Bloomington Drosophila Stock Center, BDSC# 5358), UAS-RREB1::GFP (Ming et al., 2013) were used to overexpress Oamb, Timp, Hnt, and RREB1, respectively. Oamb.K3 is the
Oamb isoform expressed in wild-type stage-14 follicle cells (Deady and Sun, 2015). \(hnt^{XE81}\) and \(hnt^{EH704a}\) are loss-of-function \(hnt\) alleles, while \(hnt^{peb}\) (BDSC# 80) is a temperature-sensitive \(hnt\) allele (Wilk et al., 2004). Animals bearing \(hnt^{peb}\) were raised at room temperature, and newly emerged adult flies were shifted to the 29°C restrictive temperature. For generating flip-out actin-Gal4 clones (Pignoni and Zipursky, 1997), \(hsFLP;;act<CD2<Gal4,UAS-RFP/TM3, Sb\) (derived from BDSC# 30558) was used to cross to \(hnt^{EP55}\) or \(hnt^{EP55}; UAS-hnt^{RNAi}\) and adult flies were heat shocked in a 37°C water bath for 45 minutes. \(UASp-GFP::act79B; UAS-mCD8::GFP\) was crossed to Gal4 lines and used to visualize Gal4 expression pattern. \(UAS-RFP\) was recombined to Gal4 drivers and used for isolating stage-14 egg chambers for \textit{ex vivo} culture. \(UAS-GCaMP5G\) (Akerboom et al., 2012) was used to visualize calcium responses in follicle cells (BDSC# 42037). Protein trap lines \(vkg::GFP^{CC00791}\) (Buszczak et al., 2007) and \(Mmp2::GFP\) (Deady et al., 2105) were used for Vkg and Mmp2 expression, respectively. Control flies for all experiments were prepared from crossing Gal4 drivers to Oregon-R.

**Ovulation assays**

Egg laying and egg-laying time analyses were performed as previously described (Deady and Sun, 2015; Knapp and Sun, 2017). Five virgin females (five-to-six days old with one day of wet yeast feeding) were placed with ten Oregon-R males in one bottle to lay eggs on grape juice-agar plates with a drop of wet yeast paste for two days in 29°C. After each day (22h in 29°C) of egg laying, grape juice-agar plates were removed and replaced with a new one. Typically, five bottles for each genotype are performed in each experiment. After egg laying, ovaries were dissected and mature follicles in female ovaries were counted. Virgin females were dissected before mating for a “pre-egg laying” mature follicle count to ensure normal oogenesis occurred. The average number of eggs laid per female per day was used to calculate the average time to lay one egg, as described previously. The egg-laying time was further proportioned into the amount of time an egg spent in the ovary (ovulation time), in the oviduct (oviduct time), and in the
uterus (uterus time) according to the distribution of females with eggs in their reproductive tract six hours after mating. For this assay, ten virgin females and fifteen males are mated in a vial with dry yeast at 29°C. Typically, two to three vials for each genotype were performed in each experiment. After the six hours of mating the flies were frozen at -80°C for approximately four minutes, and then dissected to examine the location of an egg within the reproductive tract.

**Ex vivo follicle rupture, gelatinase assay, and quantitative RT-PCR**

The ex vivo follicle rupture assay was performed as described previously (Deady and Sun, 2015). In brief, 5-6-day-old virgin females fed with wet yeast for 2-3 days were used to isolate stage-14 egg chambers in Grace's insect medium (Caisson). Within one hour, isolated mature follicles from ~10 females were separated into groups of ~30 egg chambers, then cultured in culture media (Grace's medium, 10% fetal bovine serum, and 1X penicillin/streptomycin) supplemented with 20 μM OA (Sigma), or 5 μM ionomycin (Cayman Chemical). All cultures were performed at 29°C, the same condition as flies were maintained, to enhance Gal4/UAS efficiency. One data point represents the percent of ruptured follicles per experimental group (~30 egg chambers). Data were represented as mean percentage ± standard deviation (SD).

In situ zymography for detecting gelatinase activity was performed as previously reported with minor modifications (Deady and Sun, 2015) 20-25 μg/mL of DQ-gelatin conjugated with fluorescein (Invitrogen) was added into the culture media with 20 μM OA for three hours. Mature follicles with posterior fluorescein signal were directly counted, and data represented as percent of follicles with posterior fluorescein signal. Follicles with lateral fluorescein signal, which is likely induced by damage during dissection, are not counted as Mmp2 activity, because Mmp2 is only expressed in posterior follicle cells (Deady and Sun, 2015).

For quantitative RT-PCR, total RNA was extracted from 60 stage-14 egg chambers isolated from 10 flies using Direct-zol™ RNA MicroPrep Kit (Zymo Research). cDNA synthesis, real-time PCR amplification and primers of *Oamb.K3* and *Mmp2* were described previously.
The data are presented as mean ± SEM from three biological replicates, except for RREB-1 rescue experiment, in which one single biological experiment was presented.

Immunostaining and microscopy

Immunostaining was performed following a standard procedure, including ovary dissection, fixation in 4% EM-grade paraformaldehyde for 15 minutes, blocking in PBTG (PBS+ 0.2% Triton+ 0.5% BSA+ 2% normal goat serum), and primary and secondary antibody staining diluted in PBTG. For vkg::GFP analysis, stage-14 egg chambers were first isolated from ovaries in cold Grace’s medium before fixation. Mouse anti-Hnt (1:75; Developmental Study Hybridoma Bank), mouse anti-GFP (1:2000; Invitrogen), rabbit anti-GFP (1:4000; Invitrogen), and rabbit anti-RFP (1:1000; MBL international) were used as primary antibodies, and Alexa 488, 546, and 633 goat anti-mouse and goat anti-rabbit (1:1000, Invitrogen) were used as secondary antibodies. Images were acquired using a Leica TCS SP8 confocal microscope or Leica MZ10F fluorescent stereoscope with a sCOMS camera (PCO.Edge), and assembled using Photoshop software (Adobe, Inc.) and ImageJ.

To visualize calcium response to ionomycin and octopamine, egg chambers expressing GCaMP5G and hntRNAi were isolated into an imaging chamber. Images were acquired on a Zeiss Axio Zoom microscope at 0.2 FPS, and 10 μL of ionomycin or octopamine were added to the solution after frame 5 to a final concentration of 5 μM or 20 μM, respectively. A ROI in the center of the main-body follicle cells was selected and the integrated intensity was measured. F0 was defined as the average baseline intensity (first five frames), and ΔF/F0 is reported.

Statistical analysis

Statistical tests were performed using Prism 7 (GraphPad). For comparison of more than two means, one-way ANOVA with post hoc Fisher’s Least Significant Difference test was used. For comparison of distribution, Chi square test was used except in Figure 3D and 3H, where
Fisher’s exact test was used. In addition, Z-score test was used for egg-laying time analysis in Figure 3K-L and Table 1.

ACKNOWLEDGEMENTS

We are thankful to Drs. Howard Lipshitz, Bruce Reed, Allan Spradling, Dirk Bohmann, Kyung-An Han, Andrea Page-McCaw, and Wu-Min Deng for sharing fly lines, the Bloomington Drosophila Stock Center and the Vienna Drosophila Stock Center for fly stocks, and the DHSB for antibodies. We are very grateful to Virge Kask at UConn Scientific Illustration Office for providing the illustration of Drosophila ovulation process, to the Conover, LoTurco, and Kanadia labs for sharing reagents and microscopes. We thank Drs. John Peluso and Joseph LoTurco as well as the anonymous reviewers for discussion and comments regarding the manuscript; and are grateful for Elizabeth Knapp and Wei Shen for technical support. Leica SP8 confocal microscope is supported by a NIH award (S10OD016435) to Akiko Nishiyama. J.S. is supported by the University of Connecticut Start-up fund, NIH/National Institute of Child Health and Human Development Grant R01-HD086175, and Bill & Melinda Gates Foundation.
Supplemental Figure 5-1 Egg chambers with different patterns of Hindsight expression

(A–B') Hnt (red in A-B and white in A'-B') is expressed in posterior tip follicle cells (arrowheads) in youngest stage-14 egg chambers, which have faint FC1 (A) but no FC2 (B) expression (green). (C–D'') Representative images show all three types of egg chambers with differential Hnt expression. (I) A/P-Hnt (stage-14A) egg chambers; (II) high-Hnt (stage-14B) egg chambers; (III) low-Hnt (stage-14C) egg chambers. DAPI labeling nuclei is shown in blue (A–D), or white (C'”, D'”).
Supplemental Figure 5-2 Hindsight expression pattern in stage-14 A and stage-14 B egg chambers

(A–H) Hnt expression (green in A–C and E–G) in stage-14A (A–D) and stage-14B (E–H) egg chambers with FC1 driving RFP (control, A and E) or hnt-RNAi (B–C and F–G). Quantification of Hnt expression is shown in (D) and (H). (I–P) similar as in (A–H) but using FC2 Gal4 instead.

Supplemental Figure 5-3 Hindsight depletion in stage-14 follicle cells does not affect oogenesis in virgin
(A–B) Quantification of stage-14 egg chambers in virgin females before egg laying experiment. (A) is using FC1-Gal4, while (B) is using FC2-Gal4.

Supplemental Figure 5-4 Hnt mutant mature follicles are defective for OA-induced follicle rupture.

(A–C) Representative images show hnt[peb]/+ (A), hnt[peb]/hnt[XE81] (B), and hnt[peb]/hnt[EH704a] (C) mature follicles after three-hour culture with OA. Mature follicles were isolated according to FC2 > RFP expression showing in red. Bright-field images of the egg chambers are shown in blue, and ruptured follicles are marked by white arrowheads. (D) Quantification of OA-induced rupture is shown. ***p<0.001.
Supplemental Figure 5-5 Measurement of intracellular calcium in follicle cells after ionomycin or octopamine stimulation

(A–D) Intracellular Ca2+ depicted by GCaMP5G (green in A–C’) increase in response to ionomycin stimulation in both control (A–A’) and hnt-RNAi (B–C’) egg chambers with FC2 Gal4, although hnt-RNAi egg chambers show slightly weaker response. The frames with peak GCaMP5G signal after ionomycin stimulation are shown in A’-C’. Quantification of intracellular Ca2+ level (ΔF/F0) is shown in D and the number of egg chambers analyzed is noted at the end of each trace. (E–H) Intracellular Ca2+ depicted by GCaMP5G (green in E–G’) increase in control egg chambers (E–E’) after OA stimulation but does not in hnt-RNAi egg chambers with FC2 Gal4 (F–G’). The frames with peak GCaMP5G signal after OA stimulation are shown in E’-G’. Quantification of intracellular Ca2+ level (ΔF/F0) is shown in H and the number of egg chambers analyzed is noted at the end of each trace. Also see Videos 1–6.
Supplemental Figure 5-6 Hnt depletion disrupts Mmp2 expression in posterior follicle cells

*Mmp2::GFP* (green in A-C and white in A'-C') is highly expressed in posterior follicle cells of control stage-14 egg chambers (A–A'), but is weakly detected in posterior follicle cells of *hnt-RNAi* egg chambers (B–C') with FC1 Gal4. The oocyte is outlined in cyan (A'–C'), and nuclei are labeled with DAPI and shown in blue (A–C).
Supplemental Figure 5-7 Rescue of ovulation defect in Hnt-RNAi females with Hnt overexpression

(A–D) Quantification of OA-induced follicle rupture (A, C) and the egg-laying number (B, D) from females with FC1 (A–B) and FC2 (C–D) Gal4 driving hnt-RNAi and/or hntEP55 overexpression. 

(E–L) Hnt expression (green, white in insets of outlined area) in stage-14C control (E, I), hnt-
RNAi1 (F, J), hnt-RNAi1/hntEP55 (G, K), and hntEP55 (H, L) follicles with FC1 (FC1 > RFP in red, (E–H) or FC2 (FC2 > RFP in red, (I–L). (M–P’) Hnt expression (green in M–P and white in M’–P’) in stage-10B (M–N) and stage-14B (O–P) egg chambers. Hnt is highly overexpressed in a flip-out clone with actin-Gal4 and hntEP55 (M and O) but is barely detected in the clone with actin-Gal4 and hntEP55/hnt-RNAi1 (N and P). Clone cells are marked by actin-Gal4 driving UAS-RFP expression (red) and DNA is labeled with DAPI in blue (M–P). *p<0.05, **p<0.01, ***p<0.001.

Supplemental Figure 5-8 Human RREB-1::GFP does not interfere with Hnt expression in control of hnt-RNAi egg chambers

Hnt expression is shown in yellow (A–F) and white (A’–F’), and RREB1::GFP expression is shown in green (D–F) and white (D”–F”). (A–C) Hnt expression is detected in control follicle cells (A) and is reduced in follicle cells with FC1 driving hnt-RNAi expression (B–C). (D–E) Hnt expression is still reduced in follicle cells with FC1 driving hnt-RNAi/RREB-1::GFP expression. (F) Both Hnt and RREB-1::GFP are highly expressed in follicle cells with FC1 driving RREB-1::GFP expression.
Supplemental Figure 5-9 Overexpression of GFP is not sufficient to rescue rupture defect of Hnt-RNAi follicles with FC1-Gal4.

Quantification of OA-induced follicle rupture is shown.
Table 1. The egg laying, egg distribution within the reproductive tract, and egg laying time of females with various genotypes

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Eggs laid in 2 days</th>
<th>Egg distribution in 6hr</th>
<th>Egg laying time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Eggs laid/ female/day</td>
<td>Uterus with egg (%)</td>
</tr>
<tr>
<td>UAS-dcr2/+; 44E10-Gal4/(One-R)</td>
<td>75</td>
<td>58.04 ± 1.15</td>
<td>44.07 ± 12.67</td>
</tr>
<tr>
<td>UAS-dcr2/HntRNAi1; 44E10-Gal4/+</td>
<td>75</td>
<td>37.73 ± 0.86***</td>
<td>26.03 ± 10.07</td>
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<tr>
<td>UAS-dcr2/HntRNAi2; 44E10-Gal4/+</td>
<td>75</td>
<td>31.34 ± 1.07***</td>
<td>44.58 ± 10.69</td>
</tr>
<tr>
<td>UAS-dcr2/+; 47A04-Gal4/(One-R)</td>
<td>50</td>
<td>61.60 ± 2.02</td>
<td>35.09 ± 12.39</td>
</tr>
<tr>
<td>UAS-dcr2/HntRNAi1; 47A04-Gal4/+</td>
<td>50</td>
<td>31.03 ± 1.75***</td>
<td>25.93 ± 11.69</td>
</tr>
<tr>
<td>UAS-dcr2/HntRNAi2; 47A04-Gal4/+</td>
<td>35</td>
<td>41.22 ± 1.95***</td>
<td>45.83 ± 9.97</td>
</tr>
<tr>
<td>UAS-dcr2/+; 44E10-Gal4/(One-R)</td>
<td>125</td>
<td>68.74 ± 1.08</td>
<td>45.90 ± 12.51</td>
</tr>
<tr>
<td>UAS-dcr2/HntRNAi1; 44E10-Gal4/+</td>
<td>100</td>
<td>38.14 ± 0.64***</td>
<td>20.34 ± 10.27</td>
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<tr>
<td>UAS-dcr2/HntRNAi2; 44E10-Gal4/UAS- RREB1::GFP</td>
<td>55</td>
<td>20.63 ± 1.29***</td>
<td>13.04 ± 9.73</td>
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<tr>
<td>UAS-dcr2/HntRNAi2; 44E10-Gal4/UAS- RREB1::GFP</td>
<td>90</td>
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<td>25.86 ± 11.27</td>
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<tr>
<td>UAS-dcr2/HntRNAi2; 44E10-Gal4/UAS- RREB1::GFP</td>
<td>100</td>
<td>56.66 ± 1.14**</td>
<td>32.79 ± 11.78</td>
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<tr>
<td>UAS-dcr2/HntRNAi2; 44E10-Gal4/UAS- RREB1::GFP</td>
<td>55</td>
<td>50.43 ± 2.77***</td>
<td>27.45 ± 12.25</td>
</tr>
</tbody>
</table>

All data are plotted as average ± 95% C.I.
One day is considered as 22h in 29 °C.
* P<0.05, ** P<0.01, and *** P<0.001. One-way ANOVA with post hoc Fisher’s Least Significant Difference test is used for egg laying, and Z-score test is used for egg laying time assuming normal distribution.

Corresponding videos, found online

**Video 1.** Signal of GCaMP5G driven by FC2-Gal4 in Control follicles with ionomycin stimulation (FC2 > GCaMP5G).
https://doi.org/10.7554/eLife.29887.015

**Video 2.** Signal of GCaMP5G driven by FC2-Gal4 in hnt-RNAi1 follicles with ionomycin stimulation (FC2 > GCaMP5G/hnt-RNAi1).
https://doi.org/10.7554/eLife.29887.016

**Video 3.** Signal of GCaMP5G driven by FC2-Gal4 in hnt-RNAi2 follicles with ionomycin stimulation (FC2 > GCaMP5G/hnt-RNAi2).
https://doi.org/10.7554/eLife.29887.017

**Video 4.** Signal of GCaMP5G driven by FC2-Gal4 in Control follicles with octopamine stimulation (FC2 > GCaMP5G).
https://doi.org/10.7554/eLife.29887.018

**Video 5.** Signal of GCaMP5G driven by FC2-Gal4 in hnt-RNAi1 follicles with octopamine stimulation (FC2 > GCaMP5G/hnt-RNAi1).
https://doi.org/10.7554/eLife.29887.019
Video 6. Signal of GCaMP5G driven by FC2-Gal4 in hnt-RNAi2 follicles with octopamine stimulation (FC2 > GCaMP5G/hnt-RNAi2).

https://doi.org/10.7554/eLife.29887.020
CHAPTER 6: SUMMARY OF MAJOR FINDINGS

Before the results published here, the concept of ovulation and follicle rupture in Drosophila was very poorly understood. We demonstrate that the follicle-cell layer is selectively degraded and recedes toward the anterior to expel the oocyte out of the ovary, and this process is heavily dependent upon Mmp and octopamine. We demonstrated the role for many genes required in this process: Mmp2, Oamb, Gaq, IP3R, Stim, Calmodulin, CaMKII, and Hindsight. Describing the genetic regulation and signaling patterns of ovulation is the first step in developing Drosophila to be a suitable model for ovulation.

**Conserved Enzymatic Process for Mammalian and Drosophila Ovulation**

Many years of work have attempted to demonstrate a requirement for enzymes, particularly Mmps, in ovulation. Upon the observation that intrafollicular pressure is not the ultimate cause for follicle rupture, the hypothesis that the follicular wall was weakened was investigated (Parr, 1975). Likely due to genetic redundancy (23 described Mmps in mice and humans), efforts toward this process have been unsuccessful. Analysis of expression patterns have demonstrated upregulation of Mmps along the apex of a periovulatory follicle, and this was conserved between rodents and primates (Puttabyatappa et al., 2014); however there hasn’t been a function attributed to these correlations. By using a simpler model system, we were able to genetically perturb the function of the two Mmps in Drosophila and identify that Mmp2 and not Mmp1 activity was indeed required for ovulation and female fertility. We identified that Mmp2 activity was localized to one follicle per ovary, and this follicle had a receding follicle-cell layer. We also described the fate of the follicle cells after ovulation, a question that had remained unknown in the Drosophila field. After follicle rupture, we found that the follicle-cell layer remained in the ovary, developed a yellowish pigmentation, and expressed enzymes for ecdysone synthesis; we termed the residual follicle cells as the corpus luteum.
This work led us to the hypothesis that Mmp2 activity in the posterior follicle cells caused follicle rupture and ovulation. This hypothesis provided the basis for the other chapters within this dissertation, as we had identified a requisite physiological process for ovulation and sought to identify how it was regulated. However, most importantly, this study provided evidence that Drosophila and mammalian ovulation was more conserved than previously thought.

**ADRENERGIC REGULATION OF OVULATION AND THE DEVELOPMENT OF THE FOLLICLE RUPTURE ASSAY**

We learned that Mmp2 was expressed in all stage-14 follicle cells in Chapter 2, but it was only activated in one per ovary. Therefore we sought to learn what was regulating its activation. We found Oamb expression on the stage-14 follicle cells was required for ovulation, and that stimulation of these follicles with exogenous octopamine was actually sufficient to induce follicle rupture (continued in next paragraph). Through a modification of an *in situ* zymography experiment, we found that octopamine indeed stimulates Mmp activity. This work furthered our understanding of *Drosophila* ovulation – that octopamine (which was well known to be required for ovulation, through many mechanisms) acted on the follicle cells to induce ovulation, not just the oviduct.

Beyond furthering our understanding of ovulation in *Drosophila*, the most significant aspect of this publication (in my opinion) is the development of the *ex vivo* follicle rupture assay. With the ability to genetically modify any gene of interest and directly stimulate follicles to rupture in a culture dish, this dramatically expanded the opportunity to study ovulation. As mentioned throughout the introduction chapter, ovulation *in vivo* is a very complex process and is regulated by so many variables. There wasn’t a direct way to measure ovulation, rather one would have to assimilate the results from many assays to attribute (or not) an ovulation phenotype. This assay is the first, and only, way to directly study follicle rupture.
IDENTIFICATION OF THE SIGNAL TRANSDUCTION PATHWAY OF OAMB

The overarching goal of this dissertation is to develop Drosophila as a model to study ovulation. Although we identified a ligand/receptor on the follicle cells to stimulate ovulation (octopamine/Oamb), it remains questionable whether this is a conserved signal. Results from studying adrenergic signaling in mammalian ovulation are often contradictory, therefore more work will need to be done to determine whether this is conserved to Drosophila. However, even though adrenergic signaling may remain a question in mammalian systems, intracellular signaling patterns may highlight more conservation. We identified the signal transduction pathway elicited by octopamine/Oamb as traditional Gαq/IP3R signal transduction to ultimately result in follicle rupture. Interestingly, in mammalian follicles, LH activates Gαq signal transduction as well to ultimately result in follicle rupture. Manipulation of these conserved pathways may provide useful targets for alternative contraceptives in inhibiting ovulation.

Of more general interest, however, we identified the signal transduction of Oamb, a well-studied octopamine receptor involved in many aspects of Drosophila physiology. Despite the many years of investigation into this receptor, no one has described its signal transduction. Furthermore, we described a new model wherein store-operated calcium entry can be studied. Epithelial calcium signaling is not well understood and being able to observe changes in calcium signals ex vivo and in vivo (via the abdomen cuticle) from genetic manipulations could be a new system to study these processes.

GAIN OF COMPETENCY TO RECEIVE AND RESPOND TO AN OVULATION STIMULUS

We sought to determine the coordination of the gain-of-competency to ovulate and we found the answer in a “stage-14 marker” that had been used for years to identify a stage-14 follicle. By using the expression pattern of hindsight, we were able to define three sub-stages of a stage-14 follicle: 14A, B, and C. Characteristic of a stage 14A follicle is high hindsight protein expression at just the posterior of the egg chamber, and this leads to early expression of Mmp2. Characteristic
of a stage 14B follicle is high hindsight protein expression throughout the main-body follicle cells, and this leads to expression of Oamb. Lastly, characteristic of a stage 14C follicle is one with relatively low hindsight expression, and this follicle is completely competent to receive and respond to an ovulation stimulus. We further demonstrate the genetic conservation of fly and mammalian systems by replacing hindsight with its mammalian homolog, Ras-Responsive Element Binding Protein-1 (RREB1). The role of RREB-1 in mammalian ovulation remains undetermined.

**Future Directions**

There are some outstanding questions unresolved by this thesis, ones that I am excited to see the outcome. First and foremost, the most often question I get during any presentation, *what is the force that expels the oocyte from the follicle-cell layer?* This question has been the basis for many of the undergraduate projects that I have been fortunate to supervise, however we still remain in the dark regarding this biological question. The various hypotheses we have tested address microtubules, actin/myosin, oocyte swelling, and extracellular matrix forces. To address one at a time: microtubule polymerization/depolymerization in each follicle-cell could induce collective-cell movement and guide the follicle cells toward the anterior, ultimately resulting in the separation of this layer from the oocyte. Similarly, actin/myosin contractions would also result in displacement of the follicle-cell layer: if each follicle cell squeezed to reduce the surface area it covered, it would also result in the follicle cells migrating to the anterior. Through pharmacological experiments, albeit without positive controls, work from one of our best undergraduates determined these forces weren’t causing the expulsion of the oocyte. We also chased another hypothesis: during ovulation, we noticed the oocyte increased in volume (we described this as “swelling”). If the posterior-most follicle cells had their posterior ECM degraded (as we described in the Hindsight paper) and they degraded (as we described in the Mmp2 paper), and every follicle cell remained with an intact ECM, then maybe the swelling of the oocyte caused the follicle cells
to retreat to the area of least resistance-the anterior. Through a series of pharmacology and genetic experiments, we were able to prevent oocyte swelling, however follicle rupture was still able to occur, which disproves this hypothesis. Lastly, the finding that collagen IV (Viking) degraded at the posterior of the egg chamber in an Mmp2-dependent manner, while the main-body follicle cells retained intact collagen IV, even throughout corpus luteum development, lead us to hypothesize a possible role for the ECM in squeezing the follicle cells toward the anterior. We hypothesized that the remaining intact collagen IV was interacting with perlecan to contract, causing the follicle cells to migrate toward the anterior. However, we used a collagenase to degrade the remaining Collagen IV and stimulated rupture, and unfortunately, follicles were still able to rupture, disproving this hypothesis. Follow-up work including appropriate controls, and perhaps genetic manipulation, is warranted for the actin/myosin and microtubule hypotheses. These seem most likely because the preliminarily experiments we have done thus far demonstrate that the follicle cells can migrate and induce rupture independently of the oocyte swelling and independent of the ECM (well, at least collagen IV). This project is being taken up by a junior graduate student in the Sun lab, and I am very excited to see the experiments and conclusions he is able to make about this quandary.

Another remaining question that I would be excited to learn the results from is what regulates Hnt expression in a stage 14 egg chamber; we described a very specific and distinct expression pattern of Hnt throughout the follicle cells and it would be thrilling to learn what regulates this specific expression. This question is something I believe will be solved within the next few years, as another graduate student is working on describing a suite of transcription factors that are required for the developmental transition from a stage 13 follicle to a stage 14 follicle. Through epistatic experiments to determine which transcription factors are up-stream or down-stream of regulating Hnt, I believe she will find a “master regulator” of this development. On a similar note, it would be interesting to perform ChIP-seq with Hnt and determine whether Hnt’s regulation of Mmp2 and Oamb was direct or indirect, and if it was indirect, results from the
ChIP-seq experiment may be able to identify an intermediate regulator of follicle competency.

Another interesting unresolved question is how does octopamine stimulate the follicle to induce follicle rupture and ovulation? Octopaminergic neurons have been identified to innervate the ovaries, however there is no evidence that they actually reach and synapse on the follicle cells. Results from the in vivo calcium imaging experiments from Chapter 4 suggest that octopamine does indeed reach the follicle cells to stimulate increased intracellular calcium, however how octopamine actually reaches the follicles remains a mystery. Through using modern genetic tools, such as GRASP (GFP-reconstitution across synaptic partners), we could be able to see whether octopaminergic terminals contact the follicle cells. If they don’t contact the follicle cells, then the release of octopamine on the outside of the ovarian sheath may act more as a hormone, and it may diffuse through the holes of the “patchy” ovariole sheath to reach the follicle cell layer. Regardless, it would be interesting to see how these neurons stimulate the follicle cells.

Similarly, it would be interesting to observe the firing pattern of octopaminergic neurons and how frequently they fire to cause the spontaneous increases in calcium concentrations in the follicle cells in comparison to when they generate an ovulation event. Again, through use of modern genetic tools, this could be achieved by using the in vivo calcium imaging set-up, while recording GCaMP signals from octopaminergic neurons and stage-14 follicle cells. I anticipate we would find a correlation between octopaminergic neuron activity and the spontaneous calcium increases. However, if there is not a direct and obvious correlation between these two events, that may support the hypothesis that octopamine is acting as a hormone and it is generating the spontaneous signals through slower diffusion. Even more interesting would be to learn what the signaling pattern was to cause follicle rupture. Before this experiment is performed, we would need to find a way to get females to ovulate. Once we have determined a way to cause ovulation, we can observe the follicle cell response and finally confirm the hypothesis that the high, sustained levels of intracellular calcium are ultimately what causes ovulation. Furthermore, if we could reliably get her to ovulate, we could observe the normal pattern of the octopaminergic neurons
and could recapitulate that using optogenetics to artificially stimulate ovulation and record the resultant response in the follicle cells in direct response to octopaminergic stimulation.
CHAPTER 7: LITERATURE CITED


Functional but Noncomplementing Drosophila Tyrosine Decarboxylase Genes DISTINCT ROLES FOR NEURAL TYRAMINE AND OCTOPAMINE IN FEMALE FERTILITY. J. Biol. Chem. 280, 14948–14955.


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