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FlyBook

Finishing the egg

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Gamete development is a fundamental process that is highly conserved from early eukaryotes to mammals. As germ cells develop, they must coordinate a dynamic series of cellular processes that support growth, cell specification, patterning, the loading of maternal factors (RNAs, proteins, and nutrients), differentiation of structures to enable fertilization and ensure embryonic survival, and other processes that make a functional oocyte. To achieve these goals, germ cells integrate a complex milieu of environmental and developmental signals to produce fertilizable eggs. Over the past 50 years, *Drosophila* oogenesis has risen to the forefront as a system to interrogate the sophisticated mechanisms that drive oocyte development. Studies in *Drosophila* have defined mechanisms in germ cells that control meiosis, protect genome integrity, facilitate mRNA trafficking, and support the maternal loading of nutrients. Work in this system has provided key insights into the mechanisms that establish egg chamber polarity and patterning as well as the mechanisms that drive ovulation and egg activation. Using the power of *Drosophila* genetics, the field has begun to define the molecular mechanisms that coordinate environmental stresses and nutrient availability with oocyte development. Importantly, the majority of these reproductive mechanisms are highly conserved throughout evolution, and many play critical roles in the development of somatic tissues as well. In this chapter, we summarize the recent progress in several key areas that impact egg chamber development and ovulation. First, we discuss the mechanisms that drive nutrient storage and trafficking during oocyte maturation and vitellogenesis. Second, we examine the processes that regulate follicle cell patterning and how that patterning impacts the construction of the egg shell and the establishment of embryonic polarity. Finally, we examine regulatory factors that control ovulation, egg activation, and successful fertilization.

Keywords: nutrient storage; oocyte maturation; follicle cell differentiation; eggshell; chorion gene amplification; egg shape; ovulation; egg activation; female reproductive tract; FlyBook

Introduction

The *Drosophila* female reproductive system occupies a large portion of the ventral abdomen and consists of 2 ovaries that are linked to bilateral oviducts. These bilateral oviducts are joined at the common oviduct, which in turn connects to the uterus (Fig. 1). The reproductive tract is also connected to several accessory organs, including the seminal receptacle (SR), a pair of spermathecae (SPT), and a pair of parovaria (Fig. 1).

Each ovary contains roughly 16 ovarioles, which are composed of a string of developing egg chambers wrapped by a thin layer of circular muscle sheath (Fig. 1; Hudson et al. 2008). The anterior end of each ovariole is called the germarium; this structure houses 2 germline stem cells that divide to produce cystoblasts. The cystoblasts undergo 4 rounds of division to produce 16-cell germline cysts; one cell will become the oocyte, while the other 15 will become highly polyploid nurse cells. Interestingly, the germ cell divisions occur via incomplete cytokinesis, leaving the cyst cells connected by intercellular bridges called ring canals. At the same time, follicle stem cells divide and produce daughters that establish a monolayer epithelium of ~29 cells surrounding the 16-cell germline cyst (Nystul and Spradling 2010). This assemblage then separates from the germarium as a stage-1 (S1) egg chamber or follicle.

Each egg chamber develops through 14 morphologically distinct stages (S1–S14) to reach maturity (King 1970; Spradling 1993). During this developmental process, which requires ~3 days, the nurse cells endoreplicate their DNA to facilitate mass production of maternal stores, and the oocyte grows in volume ~100,000-fold (King 1970). The oocyte itself is essentially transcriptionally quiescent (Mahowald and Tiefert 1970; Jambor et al. 2015); thus, this growth occurs in 3 ways (Fig. 2). First, during early-to-mid stages, nurse cells synthesize RNAs, proteins, ribosomes, and organelles, and they transport these materials into the oocyte through the ring canals (Theurkauf et al. 1992; reviewed by Hudson and Cooley 2002). Transport is selective; e.g. specific mitochondria are selected and amplified and then transferred into the oocyte (Lieber et al. 2019). Second, in the middle stages of oogenesis (S8–S10), the oocyte takes up lipoprotein particles to create a pool of stored materials for use by the embryo (see *Nutrient production and storage in oocytes*). The transition into S8 is tightly regulated by nutrient availability and is under the control of insulin and ecdysone signaling (reviewed by Peterson et al. 2015). Third, during S11, the nurse cells rapidly transfer all their contents into the oocyte in a process called "dumping"; they then undergo programed cell death (see *Nurse cell dumping and degradation*). This third phase of oocyte growth is coordinated

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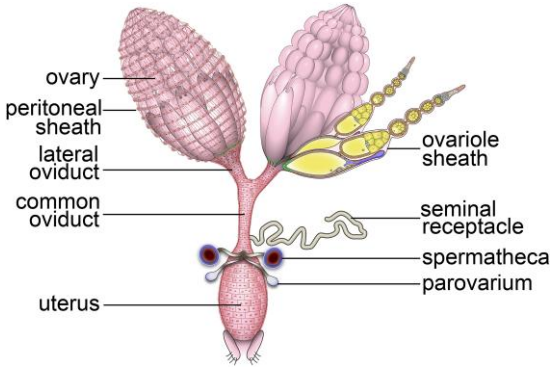

Fig. 1. Overview of the *Drosophila* female reproductive system. A schematic drawing (adapted from Deady et al. 2017) of the *Drosophila* female reproductive system shows the germline-containing ovary, which is comprised of 16 ovarioles that house the germline stem cells and developing egg chambers. The remainder of the reproductive tract (also named the lower reproductive tract) is comprised of somatic tissues such as the lateral oviducts, common oviduct, and the uterus, through which the egg passes during ovulation. The lower reproductive tract has a layer of epithelium separating the lumen and an outer muscle layer. This diagram also shows somatic structures such as the seminal receptacle, spermatheca, and parovarium, which house sperm or produce reproductive secretions that play key roles in ensuring fertilization success.

with morphological processes in the follicle cells (see *Terminal patterning and formation of the operculum and micropyle* and *Dorsal/ventral patterning and dorsal appendage formation*).

During these periods of nurse cell and oocyte growth, the somatic follicle-cell layer undergoes several cell-cycle transitions (Fig. 2). At first (S1–S5), the follicle cells divide mitotically, sometimes with incomplete cytokinesis (McLean and Cooley 2013), to form a single-cell thick layer of roughly 650–900 cells surrounding the germline cyst (Margolis and Spradling 1995; Kolahi et al. 2009; Chen, Crest et al. 2019). These estimates in follicle cell number may differ due to the use of different methods or strains for quantifying the cell-division process. At S6, the follicle cells exit the mitotic cycle and enter an endocycle in which they skip the G2 and M phases (Lilly and Spradling 1996). Finally, at the transition from S10A to S10B, the follicle cells exit the endocycle but continue to replicate DNA at 6 genomic regions, regions encoding chorion proteins needed to construct the eggshell (see *Eggshell composition* and *Chorion gene amplification*). These cell cycle switches are regulated by Notch and ecdysone signaling (reviewed by Klusza and Deng 2011; Jia et al. 2015).

The follicular epithelium plays an important role in controlling egg shape, especially during mid-oogenesis (S5–S10; see *Mechanisms of egg elongation*), and then later (S9–S14), it produces the eggshell (see *Eggshell composition* and *Chorion gene amplification*), including specializations such as the dorsal appendages (see *Dorsal/ventral patterning and dorsal appendage formation*), operculum, and micropyle (see *Terminal patterning and formation of the operculum and micropyle*). The follicle cells also exchange signals with the oocyte to establish the polarity of the egg chamber and embryo (see *Terminal patterning and formation of the operculum and micropyle* and *Dorsal/ventral patterning and dorsal appendage formation*).

Once the egg chamber matures, it releases the oocyte from the posterior ovariole into the lateral oviduct, a process called ovulation (see *Ovulation*). Shortly after ovulation, egg activation (see *Egg activation*) transforms the oocyte into a haploid cell competent for supporting embryogenesis. The egg continues moving through the common oviduct into the uterus; there, a small opening in the eggshell called the micropyle is positioned at the opening of the SR or SPT (2 types of sperm storage organs located at the oviduct–uterus junction; Fig. 1; see *Female reproductive tract secretions and reproductive success*). If the female has mated, sperm released from the sperm storage organs can enter through the micropyle to fertilize the egg. The egg is then ejected from the uterus to the outside environment, a process named oviposition. The entire sequence from ovulation to oviposition is called egg laying, which is highly coordinated by hormonal, neuronal, and reproductive tract signals and female's mating status to maximize reproductive success.

Similar to mammals, steroid hormones play critical roles in *Drosophila* oogenesis (Okamoto et al. 2023). Ecdysone and its active form, 20-hydroxyecdysone (20E), which are the only steroid hormones in *Drosophila*, were first discovered via their critical roles in insect development and metamorphosis (Truman and Riddiford 2002). Ecdysteroid hormones are produced from sterol precursors by a series of cytochrome P-450s called the Halloween genes, including *spook*, *spookier*, *phantom*, *disembodied*, *shadow*, and *shade*, to name a few (Gilbert et al. 2002; Pan et al. 2021). Once produced, 20E binds to ecdysone receptor (EcR), which forms a heterodimer with ultraspiracle (Usp) to regulate target gene expression and control developmental progression and adult physiology (King-Jones and Thummel 2005; Schwedes and Carney 2012; Swevers 2019). Although ecdysteroids have no clear roles in *Drosophila* sex determination [a process that is mediated by a series of alternative splicing events for *Sex lethal* (*Sxl*), *transformer* (*tra*), *transformer2* (*tra2*), *doublesex* (*dsx*), and *fruitless* (*fru*) (Marín and Baker 1998; Pomiankowski et al. 2004; Salz 2011)], they play more profound roles in adult oogenesis than in spermatogenesis. Specifically, ecdysteroid signaling regulates the entire progression of oogenesis from germarium to S14, including germline stem cell proliferation and differentiation; lipid accumulation and S8 quality-control check point (see *Nutrient production and storage in oocytes*); the transition from endocycle to gene amplification (see *Chorion gene amplification*); and ovulation in mature follicles (see *Ovulation*). Therefore, ecdysteroids are considered to be female sex hormones in adult *Drosophila* (Sieber and Spradling 2015). One unusual feature of ecdysone signaling in the ovary is that it acts locally on individual egg chambers or germaria (Buszczak et al. 1999; Gaziova et al. 2004; Domanitskaya et al. 2014). This local activity contrasts with its global action during larval molting and pupal metamorphosis (Riddiford et al. 2000).

Juvenile hormone (JH), another prominent insect hormone, controls metamorphosis and reproduction. In *Drosophila*, this pathway regulates follicular development, oviposition, circadian aspects of egg laying, and reproductive dormancy (Saunders et al. 1990; Easwaran et al. 2022; Kurogi et al. 2023). Nevertheless, many aspects of JH function in reproduction remain unclear.

In this chapter, we focus our discussion on the establishment of the mature oocyte, eggshell patterning and synthesis by somatic follicle cells, ovulation and egg activation, as well as the role of female reproductive tract environment on successful fertilization. Due to the page limit, we apologize for being unable to cite the works not included in this review.

Oocyte maturation

Nutrient production and storage in oocytes

Drosophila embryos undergo rapid cell division and differentiation to produce hatching larvae within a day of fertilization. To fuel this exceptional growth and development, mature oocytes are loaded with large amounts of nutrients, including carbohydrates,

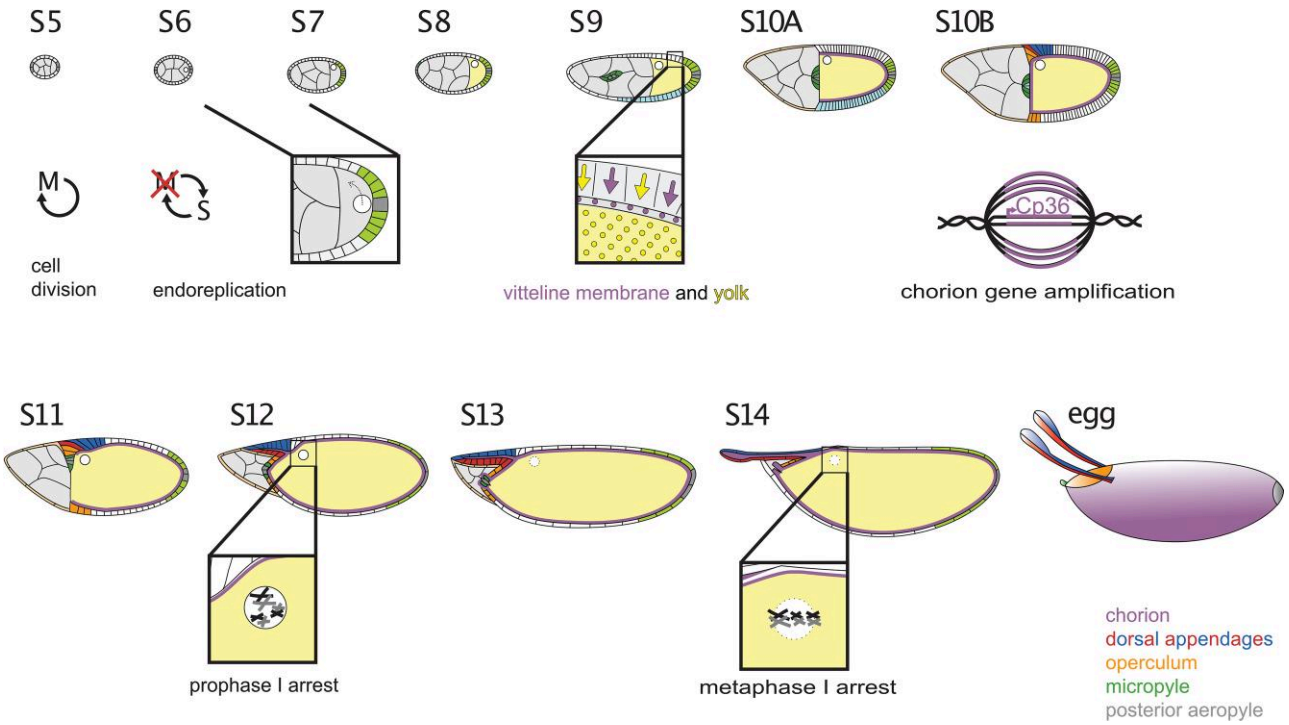

Fig. 2. Stages of oogenesis. Germline cysts enveloped by a follicular epithelium emerge from the germarium as S1 egg chambers. This chapter focuses on the events from S5 onward that are needed to finish the egg. At S5, the follicle cells complete their last mitotic cycle and enter an endoreplication cycle at S6. They shut down endocycling at the transition from S10A to 10B, but they continue to amplify regions encoding the chorion genes. At S6, a signal from posterior follicle cells induces a reorganization of the oocyte cytoskeleton, and the oocyte nucleus moves to the anterior. From S8 to S10, the follicle cells synthesize and secrete yolk proteins and vitelline membrane proteins, and from S10 to S14, they synthesize and secrete the layers and specializations of the eggshell. At S9, the border cells delaminate and migrate between the nurse cells while the stretch cells flatten. At S10B, the centripetal cells begin to ingress. At S11, the nurse cells transfer their contents into the oocyte and begin to break down; at the same time, the dorsal appendage cells wrap to make 2 tubes. From S1 to S12, the oocyte chromosomes are held in a prophase I arrest. At S13, the oocyte nuclear envelope breaks down and the chromosomes line up on the metaphase plate. When the egg chamber moves into the oviduct, the follicle cells and nurse cell remnants slough off, revealing the eggshell.

amino acids, and lipids (Tennessen et al. 2014; Sieber and Spradling 2015; Sieber et al. 2016). These nutrients are stored as large macromolecules [glycogen, yolk proteins (Yps), triacylglycerides (TAGs), and cholesterol ester (CE)] that form distinct membrane-bound particles called neutral lipid droplets and yolk spheres. Two types of yolk spheres (alpha and beta yolk spheres) exist in mature oocytes and exhibit distinct morphologies at the electron micrographic level (Mahowald 1972; Giorgi and Deri 1976; Giorgi and Jacob 1977; Giorgi et al. 1993; Papassideri et al. 2007). Alpha yolk spheres are crystalline in appearance and contain both Yps and stored lipids (Butterworth 1999; Papassideri et al. 2007). Having such a common storage depot may coordinate the mobilization of lipid and Yps and provide the raw material for early embryonic growth. In contrast, beta yolk spheres contain a mixture of Yps and glycogen. The abundance of glycogen in these structures gives the beta yolk spheres a more granular appearance and implies a role in glycogen storage (Butterworth 1999; Papassideri et al. 2007).

The process of yolk accumulation is called vitellogenesis, which starts at S8. Interestingly, these nutrients are stored in a sequential stepwise manner (Fig. 3a; Sieber and Spradling 2015; Sieber et al. 2016). Amino acids, in the form of Yps, are stored during the early stages of vitellogenesis (S8–S9; Schonbaum et al. 2000). Following Yp accumulation, large amounts of TAGs and CEs are stored in S9 egg chambers (Buszczak et al. 2002; Parra-Peralbo and Culi 2011; Sieber and Spradling 2015). After lipid storage is complete, the developing egg chamber undergoes a massive shift in metabolic state that drives a dramatic increase in stored glycogen as the egg chamber enters cellular quiescence (Sieber et al. 2016). This chronological pattern suggests that the mechanisms driving these shifts in cellular metabolism are mutually exclusive. This idea is consistent with the fact that glycolysis is required to synthesize fatty acids and that fatty acid oxidation feeds cells during the periods of gluconeogenesis. Moreover, this sequential nutrient accumulation implies that a coordinated set of developmental cues regulates the mechanisms that drive nutrient storage.

Yp production, transport, and uptake

The Yps that are stored in both alpha and beta yolk spheres provide the amino acids that feed into the tricarboxylic acid cycle and facilitate high levels of de novo protein synthesis in the early embryo (Papassideri et al. 2007). These Yps, also named Vitellogenins, are encoded by 3 *Yp* genes (*Yp1*, *Yp2*, and *Yp3*; Gelti-Douka et al. 1974). All 3 Yps contain TAG lipase superfamily domains similar to mammalian acid lipases such as pancreatic lipase (Terpstra and Ab 1988). Sequence comparisons around the catalytic site, however, indicate that Yps are unlikely to retain some TAG lipase activity.

Yps in many species commonly contain lipid-binding domains. These domains may promote the storage of both Yp and lipids required for embryonic development. *Yp3* mutant females are weakly fertile and produce oocytes with morphologically abnormal alpha and beta yolk spheres (Butterworth 1999). In contrast, certain temperature-sensitive mutations in *Yp1* and *Yp2* cause female sterility due to the formation of protein aggregates that

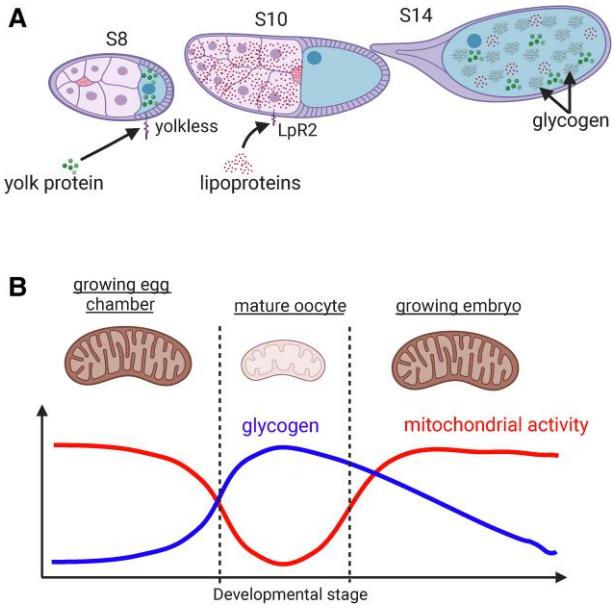

Fig. 3. Metabolic transitions drive stepwise nutrient storage during oogenesis. a) Nutrient storage occurs in a stepwise fashion during S8–S14, beginning with Yolk protein uptake during S8–S10, followed by lipoprotein uptake from S9 to S10A, and glycogen storage beginning at S10B. b) A working model of metabolic transitions shows the suppression of mitochondrial respiration that signifies the onset of MRQ (mitochondrial respiratory quiescence) as egg chambers transition from growth in earlier stages to quiescence at S10B. This suppression of mitochondrial metabolism during MRQ helps drive glycogen storage in mature oocytes for use by the developing embryo. Created with BioRender.com.

accumulate in the space between the follicle cells and oocyte and block assembly of eggshell proteins into the vitelline membrane (Gans et al. 1975; Bownes and Hobson 1980; Butterworth et al. 1992).

Many developmental inputs regulate the expression of Yps, e.g. the steroid hormone ecdysone and its active form 20E. The ecdysone level in adult females increases dramatically in the first few days after eclosion and further increases upon mating (Harshman et al. 1999). This increasing ecdysone level induces *Yp1*, *Yp2*, and *Yp3* expression in many tissues, including the fat body, heart, head, and somatic follicle cells of the ovary (Isaac and Bownes 1982). The overall effect is to increase the level of Yps in the hemolymph to meet the demands of female reproduction.

The *Yp1* and *Yp2* genes are located adjacent to each other and are divergently transcribed when ecdysone associates with EcR/Usp. EcR/Usp binds a conserved site in the shared promoter region of *Yp1* and *Yp2* (Shirk et al. 1983; Bownes et al. 1996). This clustering of functionally related genes around common binding sites is a conserved feature of nuclear receptor target genes. The GATA transcription factor encoded by *serpent* (*srp*) also binds a small enhancer region between *Yp1* and *Yp2*. Such binding is required for normal *Yp1* and *Yp2* expression in follicle cells but not fat body (Lossky and Wensink 1995). Srp, much like other GATA factors, provides tissue specificity to the expression of target genes. In this case, Srp likely functions to ensure that *Yps* are expressed in tissues with enough biosynthetic and secretory capacity to ensure that adequate levels of Yps are in circulation to support oogenesis.

Predictably, since females make the large gamete that stores nutrients, female flies specifically express Yps. This sex-specific expression is regulated in part by the transcription factor Dsx. *dsx* mutant flies display significant reductions in *Yp* gene expression and protein levels in hemolymph (Bownes et al. 1983). This defect in *Yp* expression is not merely a side effect of the intersexed nature of *dsx* mutant females. Footprinting studies demonstrate that Dsx protein can directly bind response elements in the promoters of *Yp* genes to regulate their expression (Burtis et al. 1991). While the female isoform of Dsx promotes *Yp* expression, the male isoform of Dsx inhibits *Yp* expression, providing a mechanism that ensures the female-specific expression of Yps. Interestingly, ecdysone levels are significantly higher in female flies, and the hormone behaves like a female sex hormone (Sieber and Spradling 2015) in the adult; ecdysone signaling and *dsx* likely work together to dictate the sex-specific nature of *Yp* expression.

Once produced, Yps are secreted into circulation by tissues such as the fat body. Yps pass between the follicle cells in a process called "patency," are absorbed by germ cells via receptor-mediated endocytosis, and then they are stored in yolk spheres (Richard et al. 2001; Isasti-Sanchez et al. 2021; Row et al. 2021). Both large and small alpha yolk spheres localize to the periphery of the oocyte near micropinocytic invaginations that may influence yolk sphere growth by promoting the uptake of Yps (Mahowald 1972). Yps are taken up by the Yp receptor encoded by *yolkless* (*yl*; DiMario and Mahowald 1987; Schonbaum et al. 1995, 2000). Yl belongs to the low-density lipoprotein receptor (LDLR) family of receptors and shares closest homology to the mammalian very-low-density lipoprotein receptor. During vitellogenesis, *yl* expression levels increase dramatically, and Yl protein is enriched on the oocyte membrane of developing egg chambers (Schonbaum et al. 1995, 2000). Mutants lacking Yl are female sterile and produce vitellogenic oocytes that are severely depleted of Yps (DiMario and Mahowald 1987; Schonbaum et al. 1995). These ultrastructural studies show that *yl* mutant oocytes also display a significant reduction in clathrin-coated vesicles, clathrin-coated organelles, as well as a dramatic reduction in the number of alpha and beta yolk spheres. In spite of this depletion, oocyte volume at S10B is not obviously diminished, but by S13 or S14, the egg chambers have collapsed. Intriguingly, similarities between Yl and LDLR at the amino acid level support the idea that the mechanism that drives Yp uptake and lipid storage is highly coupled. However, the mechanisms that link lipid storage and Yp uptake at a functional level remain an open question. Interestingly, recent work has implicated Yp uptake in the positioning of mRNAs such as *oskar*, suggesting nutrient uptake is coupled to germ plasm regulation (Tanaka et al. 2021).

JH also functions in yolk accumulation in many insect species, including *Drosophila* (Brookes 1969; Bell and Barth 1971; Bownes 1982; Roy et al. 2018). Interfering with JH levels in *Drosophila* females blocks vitellogenesis, possibly due to disrupted yolk production in the fat body and impaired yolk accumulation in germ cells (Giorgi 1979; Bownes 1982; Saunders et al. 1990; Bownes et al. 1996; Kurogi et al. 2023). Moreover, mutations in the JH receptor gene, *Methoprene-tolerant* (*Met*), cause a reduction in egg laying and fewer vitellogenic-stage egg chambers (Wilson and Ashok 1998), consistent with impaired yolk accumulation. The exact mechanisms by which JH acts in yolk accumulation in *Drosophila* are still unclear.

Lipid accumulation and storage

Following Yp storage, S9 egg chambers begin to absorb lipids from circulation and store large quantities of TAGs and CEs (Sieber and Spradling 2015). These forms of lipid are chemically inert storage depots for free fatty acids and free sterol that allow for safe, long-term sequestration of these metabolites during oogenesis (Parra-

Peralbo and Culi 2011). These stored lipids move through the hemolymph as lipoprotein particles, similar to humans, and are absorbed by the cell via Lipophorin receptor 2 (LpR2)-mediated uptake (Fig. 3a; Parra-Peralbo and Culi 2011). In the conventional model for lipoprotein absorption, acylglycerides in the lipoprotein particles are broken down into free fatty acids by lipoprotein lipases after endocytosis, and fatty acids are absorbed by fatty acid transport proteins such as fatty acid transport protein 1 (Fatp1) or apolipoprotein lipid transfer protein particle (Apoltp). This process, however, has not been examined molecularly in detail in the fly.

The lipophorin receptors, LpR1 and LpR2, are homologs of the mammalian LDL receptor and are both expressed at high levels on the nurse cell and oocyte membranes during S9 and S10. Mutants lacking LpR1 display little to no effect on lipid levels in the oocyte, but *LpR2* null mutant egg chambers are depleted of stored lipids and eliminated by cell death, suggesting that LpR2 is the primary lipoprotein receptor in the fly ovary (Parra-Peralbo and Culi 2011). Interestingly, LpR2 mediates lipid uptake in part by recruiting the lipid transfer protein Apoltp (Rodriguez-Vazquez et al. 2015). *LpR1*, *LpR2* double mutants arrest during S8 of oogenesis, suggesting that lipid uptake is sustained by the conventional LDL receptor-mediated uptake. These data suggest that lipid levels are tightly monitored by developing egg chambers and that lipid deficiency triggers a S8 developmental checkpoint (Parra-Peralbo and Culi 2011). The severe phenotypes exhibited by *LpR2* mutants also suggest that lipid uptake from circulation is the primary source of stored lipids in the ovary, rather than synthesis within the egg chamber itself.

As part of this process, recent work has shown that tricellular junctions in the follicle cell epithelia remodel to open small gaps between cells that allow the transport of lipids and other nutrients to the germ cells, thus facilitating nutrient storage (Isasti-Sanchez et al. 2021; Row et al. 2021). Temporal regulation is tied to the zinc finger transcription factor Tramtrak 69 (Ttk69), which controls the timing of several other processes in oogenesis (see *Eggshell gene expression occurs in temporal and spatial patterns*), and spatial regulation occurs through the signaling pathways that establish dorsoventral pattern (see *Dorsal/ventral patterning and dorsal appendage formation*; Row et al. 2021).

Developmental induction of lipid uptake during oogenesis is regulated by ecdysone signaling. Like steroid hormone produced in mammalian ovaries, ecdysone is also locally synthesized in *Drosophila* ovaries. Although the exact process is not clear, clonal analyses suggest that some enzymatic steps occur in the germline and that those products are passed to the follicle cells for further enzymatic processing (Buszczak et al. 1999; Domanitskaya et al. 2014; Ameku and Niwa 2016). During vitellogenesis, activation of the EcR/Usp receptor by ecdysone drives developing egg chambers to progress into vitellogenesis. The EcR/Usp complex induces a transcriptional shift that triggers many of the critical processes that occur during egg chamber development. Importantly, among these genes induced are many genes involved in lipid uptake, trafficking, and storage (Sieber and Spradling 2015).

EcR/Usp mediates these effects in part by regulating the activity and expression of the highly conserved adipogenic transcription factor sterol regulatory element binding protein (SREBP; Sieber and Spradling 2015). Inactivation of EcR/Usp, using temperature-sensitive mutations, causes modest reductions in SREBP and SCAP (SREBP cleavage activating protein) expression and a complete block in SREBP activation (Sieber and Spradling 2015). It is also possible that insulin signaling functions upstream of SREBP to promote lipid storage given that disruptions in the insulin pathway cause abnormalities in lipid droplet morphology (Vereshchagina and Wilson 2006). Consistent with this hypothesis, studies in mammals show that insulin/Akt signaling promotes SREBP-mediated adipogenesis (Streicher et al. 1996; Matsuda et al. 2001; Owen et al. 2012).

Previous studies have shown that SREBP is a lipid-regulated transmembrane protein that contains a transcriptional activation domain. Mammalian SREBPs are regulated by cellular sterol levels, but the *Drosophila* SREBP is regulated by fatty acids and phospholipids (Dobrosotskaya et al. 2002). Interestingly, SREBP is activated in nurse cells during vitellogenesis. Germline mutant clones for SREBP cause a dramatic reduction in stored lipid and subsequent developmental arrest (Sieber and Spradling 2015). Intriguingly, SREBP mediates much of these effects on stored lipids by regulating the expression of *LpR2* (Sieber and Spradling 2015).

These findings in *Drosophila* are consistent with numerous studies in mammals that show SREBP-1C binds and regulates the expression of the *LDL receptor* gene (Hua et al. 1993). Moreover, insulin activation has been implicated in nurse cell lipid accumulation, consistent with conserved regulation of SREBP signaling by insulin/Akt signaling (Mensah et al. 2017). In mammals, SREBPs have been studied primarily in tissues such as the liver and adipose cells. SREBPs, however, are present in yeast and *Caenorhabditis elegans* (Espenshade 2006; Todd et al. 2006; Nomura et al. 2010), which do not have these types of lipid storage organs. Moreover, studies in *C. elegans* show that disruption of the worm ortholog of SREBP (SBP-1) decreases egg production, indicating a role in female germline function (Nomura et al. 2010). Taken together, work in *Drosophila* and *C. elegans* suggests that SREBPs may have an ancient, conserved role in reproductive processes predating their systemic effects in lipogenic organs. Moreover, these studies highlight the utility of *Drosophila* as a system to dissect the role of lipid metabolism in oogenesis.

All insects, including *Drosophila*, circulate massive amounts of diacylglyceride (DAG) in lipoprotein particles. DAG can function as a signaling molecule that can activate and interact with several signaling pathways, whereas TAG is an inactive storage form for fatty acids. As a result, once absorbed by the cell, DAG must be converted into TAG to be stored. The conversion of DAG into TAG requires the esterification of a third fatty acid on to the glycerol backbone of DAG. This fatty acid esterification reaction is facilitated by a family of enzymes called diacylglyceride acyltransferases (DGATs). *midway* encodes a DGAT family enzyme that shows increasing expression throughout vitellogenesis (Buszczak et al. 2002). Mutations in *midway* cause severe depletion of stored lipids in developing egg chambers and a subsequent arrest of oocyte development at the S8 nutrient checkpoint (Buszczak et al. 2002). These arrested *midway* mutant oocytes cannot progress through development and eventually undergo cell death in S9. Recent work in S2 cells has shown that active de novo lipid synthesis drives dramatic increases in lipid droplet size (Wilfling et al. 2013). Lipid droplet size in germ cells is small, however, and despite significant increases in lipid levels in S10 of oogenesis, their size does not increase, consistent with the idea that trafficked DAG provides the majority of lipid to the egg chamber. Together, these observations are consistent with the model that Midway plays a crucial role in the final esterification and storage of these lipids. Interestingly, the lipid droplet protein encoded by *Jabba* has been implicated in lipid accumulation and the storage of specialized histone proteins (H2AV). This observation suggests that lipid accumulation may be linked to histone storage during oogenesis (Li et al. 2012; McMillan et al. 2018;

Stephenson et al. 2021). These data also suggest that lipid metabolism may be linked to genome organization and stability in the early embryo.

Glycogen storage and mitochondrial respiratory quiescence

Developing egg chambers progressing through S10–S14 undergo a massive shift in metabolic state as they enter cellular quiescence (Mermod et al. 1977; Lovett and Goldstein 1977). This shift leads to a 30-fold increase in the levels of glycogen, which is caused by active suppression of glycolysis and allowing the gluconeogenic synthesis of glucose (Fig. 3; Giorgi 1978; Papassideri et al. 2007; Sieber et al. 2016). Stored glycogen is then broken down during embryogenesis to sustain the demands of rapid growth by fueling aerobic glycolysis (Tennessen et al. 2014).

Glycogen storage is controlled by changes in insulin/Akt signaling that occur during oogenesis. Insulin/Akt signaling is active during early germline development and prevents glycogen storage (Sieber et al. 2016). As egg chambers progress into late oogenesis, insulin/Akt signaling decreases, providing a temporal developmental cue that triggers glycogen storage and may help drive the cells into quiescence. Consistent with the role of insulin/Akt signaling as a core sensor of nutritional state and germline development (Drummond-Barbosa and Spradling 2001; Richard et al. 2005; LaFever and Drummond-Barbosa 2005; LaFever et al. 2010; Hsu and Drummond-Barbosa 2011), both starvation and insulin pathway inhibition induce premature glycogen storage and mitochondrial depolarization in germ cells (Sieber et al. 2016). These observations suggest that insulin signaling functions during oocyte development as a nutrient-responsive developmental cue. In this role, insulin coordinates nutritional status with the transitions in the metabolic state that support cellular quiescence and the developmental competence of the oocyte.

Insulin signaling facilitates this metabolic transition in late oogenesis by causing a dramatic shift in mitochondrial function (Fig. 3b; Sieber et al. 2016). During early oogenesis, mitochondrial membrane potential and electron transport chain (ETC) activity are high. Beginning in S10B, however, the mitochondrial membrane potential decreases, and ETC activity drops dramatically in a process called mitochondrial respiratory quiescence (MRQ; Sieber and Spradling 2015). MRQ functions in mature oocytes to prevent nutrient loss and likely to protect the oocyte against oxidative damage before fertilization. Inhibiting insulin/Akt signaling induces a premature onset of MRQ and glycogen accumulation. These data suggest that suppressed mitochondrial activity during MRQ leads to a block in pyruvate utilization and glycolysis, thereby promoting glycogen storage in late oogenesis. Insulin/Akt signaling prevents MRQ in early germ cells by suppressing the serine/threonine kinase glycogen synthesis kinase 3 (GSK3; encoded by *shaggy*; Sieber and Spradling 2015). When Akt kinase activity decreases, GSK3 stimulates a remodeling of the ETC assembly that leads to suppressed mitochondrial activity and MRQ. During ETC remodeling, complex 1 and adenosine triphosphate (ATP)-synthase are actively disassembled. Inhibiting GSK3 causes mature oocytes to display high levels of mitochondrial activity, decreased levels of nutrients, and compromised oocyte developmental competence.

Recent work has shown that GSK3 mediates this process by phosphorylating targets in the outer mitochondrial membrane, such as the voltage-dependent anion channel (VDAC, encoded by *porin*), and induces the turnover of outer membrane proteins by the ubiquitin proteasome system (UPS). The phosphorylation of VDAC triggers a massive recruitment of the proteasome to the mitochondrial surface. This recruitment drives the remodeling of the mitochondrial proteome and the suppression of respiration (Yue et al. 2022). Interestingly, this recruitment of the proteasome to the mitochondria is highly conserved in quiescent cells in systems ranging from fungi (*Neurospora*) to human cell models of cellular quiescence, indicating this process is a highly conserved mechanism that suppresses mitochondrial metabolism and promotes glycogen storage in dormant cells (Yue et al. 2022). Moreover, there is a significant 3-fold increase in cellular UPS activity in these cells that compensates for the sequestration of the proteasome to the mitochondrial surface.

This newfound role for GSK3 in MRQ has subsequently been observed in mammalian quiescent B cells, suggesting GSK3 has a conserved role in regulating mitochondrial function in quiescent cells (Jellusova et al. 2017). Consistent with a role in late oogenesis, previous work has shown that GSK3 also functions to promote the completion of meiosis through regulation of the calcineurin pathway (Takeo et al. 2012). These data suggest that GSK3 may coordinate mitochondrial function with cellular quiescence and progression through meiosis. This hypothesis is consistent with studies in yeast and *Drosophila* that show that amino acid and glucose metabolism have similar roles in the regulation of early gametogenesis (Wei and Lilly 2014). Interestingly, insulin signaling also functions early in germ cell development to regulate mitochondrial DNA number via Myc, suggesting that insulin signaling has multiple roles in regulating germline mitochondrial function (Wang et al. 2019). Moreover, this early role for insulin signaling is likely coupled to the active selection of high-quality mitochondria that occur in germ cells very early in cyst development (Lieber et al. 2019). These mechanisms likely cooperate to ensure oocyte developmental competence.

Systemic signaling regulating nutrient storage

Considering the tremendous biosynthetic and energetic demands of oocyte production, female flies alter their metabolic state. Newly eclosed male and female flies display similar levels of TAG and glycogen. As the females mature, however, they establish an enhanced metabolic state that supports oocyte development.

Drosophila females are larger and contain roughly 2-fold more stored TAG and glycogen than seen in males (Sieber and Spradling 2015). Females store more TAG and glycogen in part by increasing their feeding rate relative to males. Indeed, metabolic sexual dimorphism is required for female fertility. Mutations and RNAi lines disrupting *lipid storage droplet 2* and *magro*, genes involved with lipid storage and digestion, cause significant reductions in female TAG levels and result in reduced egg laying (Sieber and Spradling 2015).

Females establish metabolic sexual dimorphism by increasing the levels of 20E in mature adults (Sieber and Spradling 2015). In the adult, the ovary is the primary source of steroid production, and production increases after the female mates, leading to significantly higher levels of 20E in female flies (Harshman et al. 1999; Ameku and Niwa 2016). In the female, 20E acts as a female sex hormone to control metabolic sexual dimorphism. Ectopic 20E feeding can stimulate glycogen storage and TAG accumulation in males, demonstrating that ecdysone is sufficient to establish a female metabolic state (Sieber and Spradling 2015). 20E establishes a female metabolic state by acting through EcR/USP in the central nervous system (CNS) to enhance feeding behavior either by altering the neuroendocrine axis [insulin, adipokinetic hormone (Akh), etc.] or directly modulating the neural circuit that dictates feeding in the fly. How exactly 20E establishes a female metabolic state, however, remains an open question. As discussed above, 20E is thought to be predominantly produced by individual late-stage

egg chambers. As egg production increases, 20E levels should rise and further enhance feeding when females are producing more oocytes. This feed-forward mechanism allows the female to tune female systemic metabolic state to support the number of eggs she is producing.

While sex and reproductive status can influence the female metabolic state, systemic metabolism has a tremendous impact on the regulation of oogenesis. Studies in *Drosophila* have shown that female flies fed a diet depleted in amino acids and lipids reduce egg production. Under these conditions, S10B egg chambers complete their development, but young egg chambers arrest at the S8 checkpoint, S8–S10 egg chambers die and are resorbed, and the germline stem cells stop dividing in the germarium (Drummond-Barbosa and Spradling 2001; Buszczak et al. 2002; Parra-Peralbo and Culi 2011). The S8 checkpoint is a nutrient-dependent quality control checkpoint that is under control of hormonal cues and metabolic status (Buszczak et al. 1999; Drummond-Barbosa and Spradling 2001; Parra-Peralbo and Culi 2011). This checkpoint is thought to be present in other insect species and may be a conserved aspect of oogenesis (Chapman et al. 2013). These dietary effects on oogenesis are regulated in part by reduced neuronal production of insulin-like peptides (Britton et al. 2002; LaFever and Drummond-Barbosa 2005). In response to amino acid deficiency, the neural insulin production decreases, thereby causing severe disruptions in development and reproduction (Britton et al. 2002; Rulifson et al. 2002). Under these conditions, insulin signaling functions as a systemic nutrient sensor that monitors metabolic state and adjusts growth, development, and reproductive status to match nutrient availability.

One tissue that integrates systemic metabolism and reproduction on many levels is the fat body. In *Drosophila*, the fat body combines the function of adipocytes and hepatocytes and plays a significant role in the regulation of systemic metabolism. Fat tissue provides a primary source of several yolk components for developing egg chambers. The fat body synthesizes and stores vast amounts of TAGs that are necessary for egg development. Fat body TAGs are broken down into DAGs, packaged into lipoprotein particles, and secreted to provide the predominant source of the circulating lipoprotein particles in the body. These lipoprotein particles supply the ovary with DAGs and sterols during vitellogenesis (Parra-Peralbo and Culi 2011; Palm et al. 2012). The fat body is also a significant source of Yps for developing oocytes. The fat body produces and secretes Yp1, Yp2, and Yp3 into circulation (Bownes and Hobson 1980). These circulating Yps are abundant in hemolymph, highlighting that the ovary requires peripheral tissue to meet the protein yolk requirements for oogenesis (Isaac and Bownes 1982).

Interestingly, in response to alterations in the diet, many processes change within the fat body. These processes include a diverse array of metabolic pathways including glycolysis, iron transport, and the Kennedy pathway for phospholipid biosynthesis (Matsuoka et al. 2017). Disruption of the Kennedy pathway (*eas-RNAi*) or iron transport (*Fer1HCH-RNAi*) in the fat body causes defects in germline stem cell maintenance, whereas disrupting the glycolysis/gluconeogenesis pathway causes defects in early germ cell cyst survival (Matsuoka et al. 2017). These data suggest that specific fat metabolic processes are linked to distinct aspects of oogenesis, either through the regulation of fat body-derived hormones or through the trafficking of downstream metabolites to the ovary.

The fat body also produces and secretes extracellular matrix (ECM) components, such as the type IV collagen encoded by *viking* (*vkg*) and *collagen type IV alpha 1* (*Col4a1*), that incorporate into basement membrane throughout the larval body to control organ shape (Pastor-Pareja and Xu 2011; Peng et al. 2022). As discussed below (see *Mechanisms of egg elongation*), developing egg chambers deposit collagen IV in bands around the egg chamber to help drive elongation and shape the egg (Haigo and Bilder 2011; Weaver and Drummond-Barbosa 2018).

In conjunction with this biosynthetic role, the fat body also plays an essential signaling role in regulating oogenesis. Studies of the adipokine adiponectin in *Drosophila* have shown that the adiponectin receptor AdipoR functions in the ovary to promote germline stem cell maintenance (Laws et al. 2015). Overexpression of AdipoR protects germaria from stem cell loss (Laws et al. 2015), suggesting that abnormalities in fat body signaling may contribute to the age-associated decline in fertility seen in many systems including *Drosophila*.

Nurse cell dumping and degradation

Nurse cell dumping and its regulatory mechanism

Another major source of maternal stores is the highly polyploid nurse cells, which synthesize ribosomes, mitochondria, cytoskeletal subunits, tRNAs, and other products that facilitate rapid development of the embryo. Throughout the early stages of oogenesis, some select material moves from the nurse cells on cytoskeletal tracks through intercellular bridges, the ring canals, and into the oocyte. Recent work has found that cortical dynein also transports microtubules (MTs) through the ring canals as a means to transport bulk cytoplasmic content into the oocyte (Lu et al. 2022). The vast majority of the nurse cell contents, however, is transferred extremely rapidly (20 minutes) at S11 in a process called nurse cell dumping (reviewed by Mahajan-Miklos and Cooley 1994b).

Beginning in stage 10B, the egg chamber prepares for nurse cell dumping by organizing 2 classes of actin filaments in the nurse cells. At the cortex, actin polymerization and myosin activity provide much of the mechanical force that drives the transfer of nurse cell components through the ring canals into the oocyte (Cooley et al. 1992; Cant et al. 1994; Hudson and Cooley 2002; Airoldi et al. 2011). A recent study suggests that the actomyosin contractile force is only responsible for the completion of the dumping, while the initial dumping of the most cytoplasm is mediated by hydraulic transportation (Imran Alsous et al. 2021). In addition, the large polyploid nurse nuclei are anchored in position by actin filaments to prevent these structures from blocking the ring canals and preventing the transfer of nurse cell components (Cooley et al. 1992; Tilney et al. 1996; Guild et al. 1997).

Actin also is a major structural component of the ring canal itself, and actin polymerization and crosslinking play important roles in ring canal growth and pore formation. Given that all the nurse cell-derived factors must be transported into the oocyte via ring canals, studies have focused on ring canal synthesis and maturation as a way of analyzing the regulation of transport into the oocyte (Hudson and Cooley 2002). Disruption of actin-binding proteins such as profilin (*chickadee*) and villin (*quail*), as well as actin motor proteins such as nonmuscle myosin II (*spaghetti squash, zipper*), causes defects in the nurse cell cytoskeleton, defective ring canal formation, and a block in the transfer of nurse cell cytosolic factors to the oocyte, yielding a significant reduction in oocyte size (Cooley et al. 1992; Xue and Cooley 1993; Cant et al. 1994; Knowles and Cooley 1994; Mahajan-Miklos and Cooley 1994a; Edwards and Kiehart 1996). *quail* and *chickadee* were named after birds that produce small eggs, and dumpless mutants typically produce small eggs as well. Actin-nucleating factors such as

Spire and Cappuccino facilitate the actin mesh network assembly, maintain MT polarity and structure, and help regulate actin and MT dynamics during oogenesis (Manseau and Schüpbach 1989; Dahlgaard et al. 2007; Liu et al. 2009; Yoo et al. 2015).

The dynamic changes in the actin cytoskeleton are regulated by conserved kinases such as Rho kinase (Rok) and protein kinase N (Pkn). Deleting either of these genes in developing germ cells yields S10 egg chambers that display disorganized actin bundles and defective nurse cell dumping. Pkn antagonizes actomyosin activity in S10 egg chambers and, due to excessive actomyosin contractility, *Pkn* mutants actually delay nurse cell dumping (Ferreira et al. 2014). In contrast, Rok functions in developing egg chambers to promote the formation of actin bundles before dumping (Verdier et al. 2006). In particular, mutant egg chambers lacking Rok display a dramatic reduction in the actin bundles that anchor the nurse cell nuclei. This lack of anchoring leads to a severe dumpless phenotype due to nurse cell nuclei obstructing the ring canals.

Nurse cell dumping is also regulated by a class of active lipid-signaling molecules called prostaglandins. Prostaglandins are synthesized from essential fatty acids through either the cyclooxygenase pathway or the lipoxygenase pathway. Disrupting cyclooxygenase-mediated prostaglandin biosynthesis in *Peroxinectin-like* (*Pxt*) mutants leads to defects in egg chamber maturation and severe defects in actin bundle organization (Tootle and Spradling 2008). Interestingly, prostaglandins control actin bundle formation specifically in mid-oogenesis, suggesting a temporally defined role for these molecules (Spracklen et al. 2014). Although prostaglandins mediate these effects on developing germ cells via the actin-bundling protein fascin (Groen et al. 2012; Kelpsch et al. 2016), the mechanism for how prostaglandins affect fascin function remains unknown (Spracklen et al. 2019).

Nurse cell dumping occurs simultaneously with other developmental processes such as chorion gene amplification, centripetal cell migration, and dorsal/ventral patterning. The signals that coordinate these processes, however, remain unclear.

During oogenesis, MTs play key roles in transporting factors that are important for oocyte specification, axis determination, and egg chamber patterning (reviewed by Riechmann and Ephrussi 2001). During S10B, however, the MT network disassembles and short, subcortical MTs form that drive ooplasmic streaming until S13. This ooplasmic streaming ensures adequate mixing of the oocyte cytoplasm during nurse cell dumping. At the same time, ribonuclear protein (RNP) complexes essential for establishing embryonic polarity are captured and anchored at the poles (Forrest and Gavis 2003; Weil et al. 2006; Sinsimer et al. 2011). This phenomenon provides an intriguing system to examine how the cytoskeleton impacts biophysical processes in the cytosol (reviewed by Quinlan 2016).

Nurse cell breakdown

Once nurse cells have transferred their cytosol to the oocyte, their cellular remnants are broken down. Nurse cell turnover requires apoptosis, autophagy, and phagocytosis (reviewed by Lebo and McCall 2021), but many steps in this process remain unclear. The field commonly uses nurse cell nuclear degradation as a readout of the progress of nurse cell breakdown (Yalonetskaya et al. 2020). Nuclear breakdown begins in S12 and is associated with the activation of apoptotic caspases (Peterson and McCall 2013). During S13, nurse cell nuclei become highly acidic and are strongly TUNEL-positive (Terminal deoxynucleotidyl transferase dUTP nick end labeling), suggesting the presence of numerous DNA breaks (Peterson et al. 2003, 2007; Peterson and McCall 2013). Recent work has shown that mouse ovaries also produce nurse cells that support oocyte development and are eliminated, suggesting that nurse cell death is a very highly conserved aspect of oogenesis (Niu and Spradling 2022).

Unlike many other cell death processes, the apoptotic machinery during nurse cell death is not activated by somatic apoptotic inducers Reaper, Hid (Head involution defective), and Grim (Foley and Cooley 1998). Instead, it is activated by degradation of the *Drosophila* inhibitor of apoptosis Bruce (BIR repeat containing ubiquitin-conjugating enzyme) (Nezis, Shravage, Sagona, Johansen et al. 2010). Furthermore, mutating the key apoptotic caspases encoded by *Strica* (*Ser/Thr-rich caspase*), *Dronc* (*Death regulator Nedd2-like caspase*), *Drice* (*Death related ICE-like caspase*) and *Dcp-1* (*Death caspase-1*) yield oocytes where 1–2 nurse cell nuclei are retained by the mature egg chamber (Baum et al. 2007). Similar mild defects were obtained by overexpressing the apoptotic inhibitors p35 and DIAP (Death-associated inhibitor of apoptosis). This mild phenotype shows that the turnover of nurse cell nuclei is not a strictly apoptotic process and that nurse cell degradation is a unique form of programed cell death.

Consistent with the idea that other processes are required for nurse cell breakdown, autophagy also has a role in nuclei degradation (Bass et al. 2009; Nezis, Shravage, Sagona et al. 2010) and is induced in conjunction with the activation of apoptotic caspases (Peterson and McCall 2013). Mutations that disrupt the autophagy pathway display mild phenotypes, with a fraction of egg chambers containing a small number of persistent nurse cell nuclei. Moreover, disrupting both autophagy and apoptosis in mature germ cells does not enhance the phenotype in the ovary, suggesting that nurse cell death does not require conventional cell death mechanisms. Consistent with this idea, mutations in genes that encode DNaseII and vacuolar-type H^+-ATPases (V-ATPases) impair nurse cell turnover, suggesting that enzymatic breakdown of nucleotides and extracellular acidification by lysosomal machinery are critical for nurse cell turnover (Bass et al. 2009; Mondragon et al. 2019). Nevertheless, it remains unclear how these processes biochemically contribute to the breakdown of nurse cells.

Interestingly, phagocytosis of dying nurse cells plays an essential role in their clearance (Cavaliere et al. 1998; Foley and Cooley 1998; Nezis, Shravage, Sagona et al. 2010; Santoso et al. 2018). The phagocytic clearance of apoptotic cells requires specific engulfment receptors to recognize the apoptotic cells; recognition activates at least 2 parallel and conserved cell death (CED) pathways (CED1/CED6/CED7 and CED2/CED5/CED12) to reorganize the cytoskeleton in the nonprofessional phagocytes (stretch follicle cells) and engulf the apoptotic cells (Shklover et al. 2015a, 2015b). The phagocytic receptor Draper (CED-1) and the integrin alphaPS3 (*scab*) are expressed in stretch follicle cells during S12–13 as nurse cells breakdown. Draper works in parallel with alphaPS3, which is thought to function through CED12 signaling, to facilitate nurse cell degradation. As a result, *draper/scab* double mutants exhibit a substantial block in nurse cell nuclei breakdown. Draper and aPS3/integrin/CED12 signaling induce the nuclear acidification that is required for the breakdown of nurse cell nuclei (Timmons et al. 2016, 2017). While these phenotypes are consistent with the known role of phagocytosis in clearing cellular debris, it remains unclear how follicle cell phagocytosis impacts the germ cell autonomous aspects of the nurse cell breakdown (Bass et al. 2009; Timmons et al. 2016). Moreover, while insulin, TOR (target of rapamycin), and JNK (Jun kinase) signaling have all been implicated in the regulation of nurse cell turnover at earlier stages of oogenesis (see below and *Lipid accumulation and storage*), it remains unclear if these pathways coordinate

the cell autonomous and noncell autonomous aspects of nurse cell turnover during oocyte maturation. Interestingly, Draper, insulin/TOR, and JNK have all been implicated in other instances of developmentally programed cell death, suggesting that studies of nurse cell death will provide broad insights into development and tissue homeostasis (Tracy and Baehrecke 2013). While nurse cell degradation is a novel form of programed cell death, it remains unclear how germ cell turnover varies from traditional apoptotic or autophagic cell death at a mechanistic level.

In addition to the programed developmental nurse cell death, defects in germ cell physiology and environmental stress can trigger a quality-control check point during S8 of oogenesis. Under these circumstances, vitellogenic egg chambers undergo cell death to prevent suboptimal eggs from progressing further in development; this cell death also allows recovery of nutrients contained within those egg chambers (Pritchett et al. 2009; Jenkins et al. 2013). In contrast to programed developmental germline cell death, starvation-induced egg chamber death relies on the apoptotic machinery, including factors such as Dcp-1. Simple overexpression of apoptotic caspase inhibitors such as DIAP causes a substantial block in egg chamber death in response to starvation (Hou et al. 2021). Autophagy also functions in parallel to facilitate this form of cell death. Like developmental cell death, once egg chamber death is initiated, the follicle cells mediate the phagocytic clearance of the dying cyst via the *draper/ced-1* pathway. This process has been discussed at length in several reviews (Buszczak and Cooley 2000; Pritchett et al. 2009; Jenkins et al. 2013). Overall, *Drosophila* oogenesis provides an excellent system to examine the mechanistic differences in developmental and damage-induced forms of cell death. Studies on these regulatory events may provide critical insight into how these processes could be co-opted to prevent the aggressive ectopic growth seen in cancer.

Eggshell production

The *Drosophila* eggshell is an outstanding model for studying cell and developmental biology. Section *Eggshell composition* describes how studies on the eggshell have revealed structural features of the ECM and given insight into its synthesis program (Waring 2000; Cavaliere et al. 2008). Section *Chorion gene amplification* compares chorion gene amplification and its cell cycle regulation with metazoan DNA replication and highlights the conserved factors that regulate these processes (Tower 2004; Klusza and Deng 2011). Sections *Terminal patterning and formation of the operculum and micropyle* and *Dorsal/ventral patterning and dorsal appendage formation* outline the reciprocal signaling pathways between germ cells and follicle cells that lay the foundation for establishing the anterior/posterior (A/P), dorsal/ventral (D/V), and terminal regions of the embryo, thus determining the overall body plan of the fly (Merkle et al. 2020). These sections also examine how these same patterning signals specify follicle cell subtypes that create specialized eggshell structures required for fertilization, embryonic development, and larval hatching (Montell et al. 2012; Duhart et al. 2017; Osterfield et al. 2017; Horne-Badovinac 2020). Section *Mechanisms of egg elongation* describes how the follicle cells that secrete the eggshell also determine the overall shape of the egg (Cetera and Horne-Badovinac 2015). Thus, in this section, we describe the foundational work in *Drosophila melanogaster* that established the eggshell as a premier investigative system.

Eggshell composition

The eggshell protects the embryo by preventing dehydration and facilitating gas exchange (Hinton 1981). Two main types of proteins provide structure to the eggshell, vitelline membrane and chorion proteins (reviewed by Waring 2000; Cavaliere et al. 2008), but the construction of the eggshell exhibits a surprising interplay between these 2 constituent classes (Pascucci et al. 1996; Mauzy-Melitz and Waring 2003). The abundance of the vitelline membrane and chorion proteins, and the sex-specific nature of their expression, made analyses of these proteins highly attractive during the early molecular era (e.g. Petri et al. 1976; Waring and Mahowald 1979; Spradling and Mahowald 1980; Spradling et al. 1980; Osheim and Miller 1983; Higgins et al. 1984; Mindrinos et al. 1985; Osheim et al. 1988).

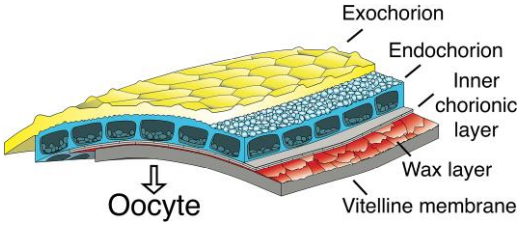

Fig. 4. Composition of the Eggshell. Moving from oocyte proximal (bottom) to external surfaces (top), the eggshell consists of a vitelline membrane (thick, solid), wax layer (thin, scalloped), inner chorionic layer (thin, brick-like), endochorion with its floor, roof, and pillars (pebbly windows), and the exochorion (surface craters). (Redrawn with permission from Margaritis et al. 1980.) See also Turner and Mahowald (1976) and Margaritis (1986) for more detail.

The eggshell consists of the vitelline envelope and chorion

Scanning and transmission electron microscopy studies demonstrate that the eggshell is composed of 5 layers (Fig. 4; reviewed by Waring 2000; Cavaliere et al. 2008). The inner 2 layers are grouped together as the vitelline envelope, while the outer 3 layers are called the chorion.

The inner most layer, the vitelline membrane, consists of 6 major and numerous minor proteins that are secreted by the columnar follicle cells during S9–S10 (Supplementary Table 1). In subsequent stages, the cleavage of 2 of the most abundant proteins, Vm26Aa and Vm26Ab, together with a 2-step crosslinking process, mediates the formation of a 300-nm envelope that serves as a barrier against the passage of macromolecules (Fargnoli and Waring 1982; Mindrinos et al. 1985). Associated with the vitelline membrane is a thin wax layer that prevents desiccation of the embryo. This layer is produced by the follicle cells in S10–S12, but its composition is unknown (Papassideri et al. 1993).

Moving radially outward into the chorion, the next layer is the inner chorionic layer (Fig. 4); it consists of glycoproteins secreted by the follicle cells beginning at S12. These proteins self-assemble into a 40-nm thick crystalline lattice (Akey and Edelstein 1987; Papassideri and Margaritis 1996). During eggshell synthesis, the inner chorionic layer holds peroxidases that covalently link chorion proteins at the end of oogenesis (Margaritis 1985; Konstandi et al. 2005). Other hypothesized functions for the inner chorionic layer are to anchor the vitelline membrane to the outer chorion layers during hatching and to regulate embryonic water content post egg-laying (Papassideri and Margaritis 1996).

Exterior to the inner chorionic layer is the endochorion, an architecturally sophisticated structure composed of floor, pillars, and roof (Fig. 4). The endochorion, which is 500–700 nm thick, contains 6 major eggshell proteins and over a dozen minor proteins (Supplementary Table 1). Synthesis begins in S10 with the production of Cp36 and Cp38; it then peaks in S12 with the addition of 4 lower molecular weight proteins, Cp15, Cp16, Cp18, and Cp19

(Petri et al. 1976; Waring and Mahowald 1979; reviewed by Waring 2000; Cavaliere et al. 2008). Based on EM immunostaining, it is likely that the chorion proteins are distributed throughout the floor, pillars, and roof; that is, the specific substructures of the endochorion are not formed by unique proteins (Pascucci et al. 1996; Noguerón et al. 2000). Intriguingly, assembly of the endochorion involves transient storage of eggshell proteins within the vitelline membrane (Pascucci et al. 1996).

The outermost eggshell layer, the exochorion, consists of a loose, polysaccharide-rich matrix containing an acidic, alcian blue-positive component, presumably glycosaminoglycan (Fig. 4). This network of protein and carbohydrate forms hexagonal or pentagonal ridges protruding from the main surface of the eggshell, mirroring the shapes of the apical surfaces of the follicle cells that secreted the eggshell layers (Turner and Mahowald 1976; Margaritis et al. 1980).

In addition to these discrete layers, the eggshell has specialized structures: a posterior aeropyle and 2 anterior dorsal appendages, which facilitate gas exchange for the developing embryo; an anterior ventral micropyle, which enables sperm entry; and an anterior operculum and collar, which act like a trap door to facilitate larval hatching (Fig. 2; Duhart et al. 2017). We discuss these specializations below in the sections *Terminal patterning and formation of the operculum and micropyle* and *Dorsal/ventral patterning and dorsal appendage formation*.

Several recent studies have exploited high-throughput technologies to identify additional components relevant to eggshell production (Supplementary Table 1). These studies employed genetic, gain-of-function screens (Khokhar et al. 2008); mass spectrometry of eggshell peptides purified from late-stage egg chambers (Fakhouri et al. 2006); microarrays and array-based comparative genomic hybridization to analyze DNA from fluorescent-activated cell sorting (FACS)-sorted follicle cells (Claycomb et al. 2004; Kim et al. 2011); or microarrays to determine genome-wide transcript levels in hand-dissected egg chambers (Tootle et al. 2011). These efforts identified potential new structural components of the eggshell as well as dozens of other genes that likely regulate chorion production or assembly. They also provided markers to facilitate cluster analysis for a single-cell RNA-seq study for assessing the development of the ovary (Jevitt et al. 2020). This scRNA-seq analysis complemented and built upon decades of work analyzing gene expression in follicle cells.

Eggshell gene expression occurs in temporal and spatial patterns

The columnar follicle cells (those lying over the oocyte) follow a precise temporal program to synthesize the major components of the eggshell: during S8–S10, they produce the vitelline membrane proteins; from S10B to S12, they make the higher MW proteins Cp36 and Cp38, and from S12 to S14, they produce the lower MW proteins Cp15, Cp16, Cp18, and Cp19 (reviewed by Waring 2000; Cavaliere et al. 2008). This timing is consistent with the construction of the eggshell layers in zones (Margaritis 1986), but the process is not so simple. For example, both Cp36 and Cp18 reside transiently in the vitelline membrane and are released over time to build up outer layers (Trougakos and Margaritis 1998a). By the end of oogenesis, however, almost all the major proteins are uniformly distributed throughout their respective eggshell layers (reviewed by Waring 2000). Two known exceptions, Vm32E protein and cleavage products of Dec, redistribute at S14, but the functional significance of these movements is not clear (Noguerón et al. 2000; Andrenacci et al. 2001). Two new strains carrying fosmid constructs expressing superfolder green fluorescent protein (GFP)-tagged chorion proteins, Cp18-sfGFP and Cp7Fc-sfGFP, should provide valuable tools for further exploring eggshell synthesis and assembly (Sarov et al. 2016).

Spatially, the major eggshell proteins are present throughout their respective layers, and indeed, genes encoding the late major chorion proteins are expressed uniformly in all columnar follicle cells (Parks and Spradling 1987). The early eggshell genes, however, achieve this homogeneous distribution by using a complex series of spatiotemporal gene expression patterns (Parks and Spradling 1987). For example, *Cp36* and *Cp38* transcripts appear first in follicle cells residing in the dorsal anterior region over the oocyte and later in the other columnar follicle cells.

Analysis of the *Cp36* regulatory region led to the insight that an overall uniform pattern of expression can occur by use of many, redundant, spatially restricted enhancers rather than a single regulatory element that specifies "house-keeping" gene expression (Tolias and Kafatos 1990; Tolias et al. 1993). The *Vm32E* gene, which unlike other vitelline membrane genes is expressed only at S10, exhibits similar complexity (Cavaliere et al. 1997). Its spatial regulation stems from epidermal growth factor (EGF) and bone morphogenetic protein (BMP) signaling, which together define dorsal anterior eggshell structures (Bernardi et al. 2006, 2007), and its temporal regulation is determined in part by steroid signaling (Bernardi et al. 2009; see below).

Hundreds of other eggshell factors exhibit spatiotemporal regulation (Claycomb et al. 2004; Fakhouri et al. 2006; Tootle et al. 2011) consistent with the patterns exhibited by unique cell types within the follicular epithelium (Yakoby, Bristow et al. 2008). It is not clear, however, whether these patterns are functionally important or simply reflect the evolution of enhancer elements.

The pioneering studies on vitelline membrane gene and chorion gene expression revealed fundamentals of gene regulation, yet relatively little is known about the transcription factors that mediate these outcomes. Molecular or genetic analyses implicate 3 proteins in this process: Chorion factor 1 (now called Usp), Chorion factor 2 (Cf2, Shea et al. 1990; Christianson and Kafatos 1993), and Tramtrack (French et al. 2003).

Usp is a member of the steroid family of nuclear receptors and is the *Drosophila* ortholog of retinoid X receptors in vertebrates (Oro et al. 1990). Usp forms heterodimers with various EcR isoforms (Riddiford et al. 2000; Yamanaka et al. 2013) and thereby plays a prominent role in regulating major transitions in the ovary (see *Nutrient production and storage in oocytes* and *Ovulation*; reviewed by Schwedes and Carney 2012; Bellés and Piulachs 2015; Swevers 2019). Although Usp binds the *Cp15* gene ~60 bp upstream of the start site for transcription (Shea et al. 1990), its role in regulating gene expression is clouded by the observation that a dominant negative form of its binding partner disrupts chorion gene amplification (Hackney et al. 2007).

The *Cf2* gene encodes 2 distinct zinc-finger proteins through alternative splicing; analyses of their DNA binding preferences demonstrated that each zinc finger recognizes a specific trinucleotide sequence, thus revealing a key insight into structure–function relationships of this entire class of proteins (Gogos et al. 1992; Hsu et al. 1992). Cf2, which binds upstream of the *Cp15* TATA box, responds to patterning signals (see *Dorsal/ventral patterning and dorsal appendage formation*) and helps establish cell fates along the dorsal-ventral axis (Hsu et al. 1996; Mantrova and Hsu 1998).

tramtrack also encodes 2 distinct zinc-finger proteins. The 69-kD isoform, Ttk69, regulates expression of *Cp15*, *Cp18*, and *Cp36* and possibly other factors that ensure the integrity of the vitelline membrane (French et al. 2003). In addition, Ttk69

mediates a key temporal transition in the formation of the dorsal eggshell structures by arresting dorsal appendage tube closure and inducing tube elongation (Boyle et al. 2010; Peters et al. 2013). These functions are independent of Ttk69's earlier role in coordinating cell cycle switches in the follicular epithelium (Sun et al. 2008; Boyle and Berg 2009; Huang et al. 2013).

The pleiotropic roles of these transcription factors present challenges for studying their function in late stages of oogenesis. Nevertheless, the application of modern genomic, computational, and comparative methods could identify binding sites, cofactors, and additional regulatory components that coordinate the spatial and temporal programs associated with eggshell synthesis (Papantonis et al. 2015).

Genetic and molecular analyses connect structure with function

Most of our understanding of eggshell proteins is based on genetic studies coupled with biochemical, molecular, and immunological analyses (reviewed by Waring 2000). For example, mutants lacking the major early chorion proteins Cp36 or Cp38 fail to form the elaborate structures of the endochorion, and these females are sterile (Digan et al. 1979; Bauer and Waring 1987; Velentzas et al. 2016, 2018). Similarly, eggs lacking the major vitelline membrane protein Vm26Ab are flaccid, and they collapse shortly after being laid due to defects in the stability of the entire eggshell (Savant and Waring 1989; Pascucci et al. 1996; Manogaran and Waring 2004; Wu et al. 2010).

From these studies, the *defective chorion* (*dec*, previously named *dec-1*) gene stands out as playing a key role in mediating the proper assembly of the eggshell (Bauer and Waring 1987; Komitopoulou et al. 1988; Noguerón et al. 2000; Mauzy-Melitz and Waring 2003). Alternative splicing produces 3 Dec proteins (Hawley and Waring 1988; Waring et al. 1990) that are secreted into the vitelline membrane, where they are later cleaved (Noguerón and Waring 1995). Some processed products are thought to mediate the construction of the floor, pillars, and roof of the endochorion, while others remain in the vitelline membrane or are taken up by the oocyte (Noguerón et al. 2000; Mauzy-Melitz and Waring 2003). Discovering how these proteins create and maintain the stability of the eggshell remains a challenge in the field.

Although Dec proteins are minor constituents of the eggshell, their functions are so important that local loss of gene products in mosaic clones creates abnormal imprints in the eggshell. This feature has made it possible to use recessive mutations in *dec* as a marker for a cell's genotype, connecting function in the follicle cells with local patterning events in the embryo, long after the follicle cells have sloughed off (Nilson and Schüpbach 1998). Similarly, mutant *dec* transgenes that lack internal coding regions produce products that interfere with endogenous Dec processing; these proteins cause dominant female sterility (Spangenberg and Waring 2007), again creating a useful tool for mosaic analyses (Lachance et al. 2009).

Several other genes encode proteins that contribute to the integrity of the eggshell, including *nudel*, *female sterile (1) M3* [also called *fs (1) polehole*], *female sterile (1) Nasrat*, and *closca* (Catalan for "turtle shell") (Degelmann et al. 1990; Hong and Hashimoto 1996; LeMosy and Hashimoto 2000; Cernilogar et al. 2001; Ventura et al. 2010). These genes were originally identified in screens for embryonic patterning mutants, but the dual nature of their protein products became apparent as investigators generated allelic series that affected additional domains or that created null mutations (see *Terminal follicle cells initiate head and tail formation in the embryo*). Intensive studies attempting to clarify the signaling pathways between the eggshell and embryo predicted the existence of another key protein, Vitelline membrane-like (Vml), and biochemical purification eventually led to its discovery (Zhang, Stevens et al. 2009).

Eggshell production is sufficiently well understood that it now serves as a powerful model for testing drug toxicity in vivo (Keramaris et al. 2020). Nevertheless, much work remains to determine the roles of the dozens of newly identified eggshell components and to ascertain the mechanisms that assemble this beautiful structure.

Chorion gene amplification

To produce the eggshell, *Drosophila* and many other insects must synthesize a large amount of chorion protein quickly (~6 h in flies). The silkmoth, *Bombyx mori*, has solved this problem by duplicating its eggshell genes, so that a single genomic region encodes over 100 related proteins (Chen et al. 2015). In contrast, *D. melanogaster* follicle cells amplify their few eggshell genes.

Numerous studies demonstrate that chorion gene amplification in *Drosophila* employs the same mechanisms that regulate DNA replication in higher eukaryotes. Using cytological, molecular, and genomic tools, investigators have measured the extent of DNA amplification, identified distinct cis elements that mediate amplification, and discovered transacting factors that facilitate the process (reviewed by Calvi and Spradling 1999; Tower 2004; Claycomb and Orr-Weaver 2005; Nordman and Orr-Weaver 2012). While early studies focused on identifying the regulatory regions that enable amplification (Amplification Control Element and oriBeta; reviewed by Tower 2004), later studies have concentrated on the molecules that allow escape from the normal cell cycle inhibition of re-replication. Complementary work has revealed the signaling pathways that initiate this cell cycle transition (reviewed by Klusza and Deng 2011).

Since DNA replication is featured in another Fly Book chapter (Hua and Orr-Weaver 2017), here we summarize the basic mechanism of chorion gene amplification, including its regulation and impact on eggshell synthesis.

Cell cycle changes allow DNA amplification at 6 sites

Chorion gene amplification occurs in the context of an altered cell cycle. That is, in young egg chambers, follicle cells divide mitotically (Fig. 2), but at S6, they enter an endocycle in which they replicate their DNA in the absence of either nuclear or cellular division. Thus, the follicle cells have already blocked mitosis and modified other aspects of the cell cycle to allow origin reinitiation without cell division (Klusza and Deng 2011).

By S10, the follicle cells have undergone 3 rounds of DNA replication and achieved a ploidy value of 16C (Lilly and Spradling 1996). At this time, ecdysone signaling in conjunction with the downregulation of Notch activity triggers major changes in follicle cell behavior, including a second switch in the cell cycle (Hackney et al. 2007; Sun et al. 2008; Boyle and Berg 2009; Huang et al. 2013; Ge et al. 2015).

At this transition from S10A to S10B, the follicle cells exit the endocycle but continue to replicate DNA at 6 genomic regions. The region containing the X-linked chorion cluster (*Drosophila* Amplicon in Follicle Cells, DAFC-7F) amplifies 16-fold, and the region containing the third chromosome chorion cluster (DAFC-66D) amplifies 60-fold (Spradling and Mahowald 1980; Spradling 1981). The 4 other regions, DAFC-22B, DAFC-30B, DAFC-34B, and DAFC-62D, also amplify but to a more modest level of 4- to 6-fold (Claycomb et al. 2004; Kim et al. 2011).

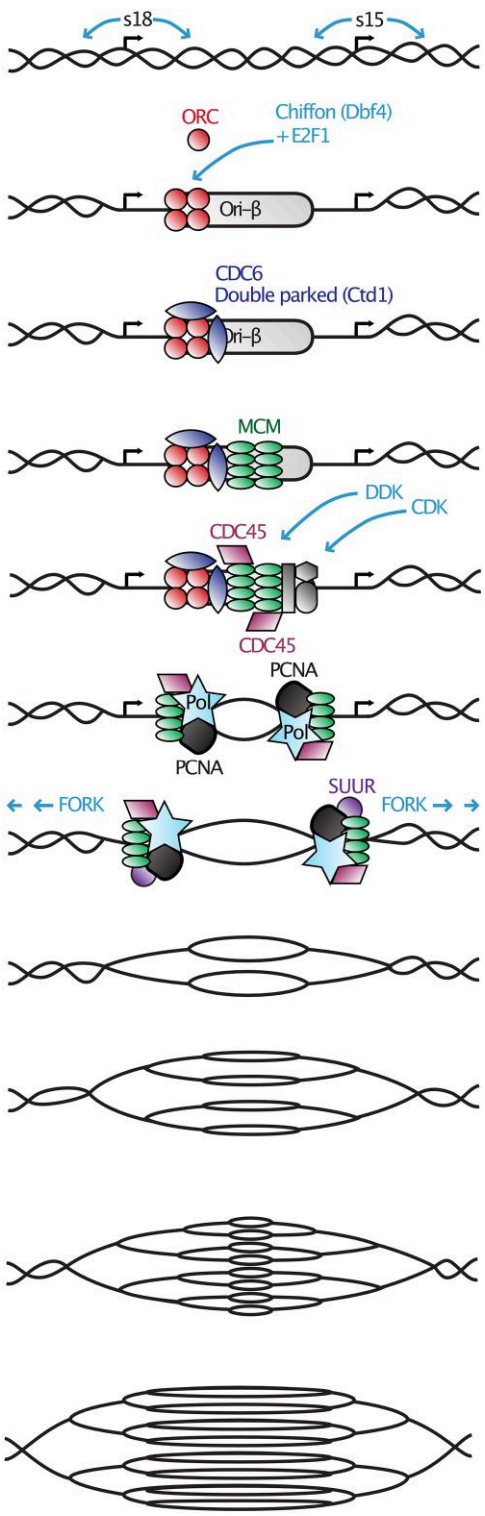

Fig. 5. Chorion gene amplification. Six locations in the genome amplify the DNA encoding major and minor chorion proteins. Shown here is the portion of DAFC-66D that contains *s18* and *s15*, genes encoding the late chorion proteins Cp18 and Cp15. DNA replication initiation occurs at discrete origins associated with open areas of chromatin, which are indicated by curved arrows bracketing each gene. The winged helix-turn-helix E2F transcription factor and the Dbf4-like zinc finger protein encoded by *chiffon* (so named for the translucent eggshells of mutants; Landis and Tower 1999) facilitate the binding of origin recognition complex (ORC), which in turn recruits several additional winged helix-turn-helix proteins (Cdc6, double-parked/Cdt1) and the mini-chromosome maintenance (MCM) DNA helicase complex, allowing association of the helicase CDC45.

(continued)

When the follicle cells transition from the endocycle to amplification, replication is shut down in most regions of the genome, probably through persistent expression of cyclin E (Calvi et al. 1998). How these 6 amplicons escape this regulation and continue to replicate their DNA is not clear (Kim and Orr-Weaver 2011), although ecdysone receptor binding might play a role (Kohzaki et al. 2020).

Chorion gene amplification is an elegant model to study metazoan DNA replication

Because chorion gene amplification is temporally regulated, replication initiation and elongation are synchronized, making this process an ideal context to study the phases of DNA replication (Claycomb et al. 2002; Kim et al. 2011). Reinitiation occurs at discrete origins [Fig. 5; e.g. oriβ, which contains an autonomously replicating sequence (ARS) consensus sequence] and is associated with open areas of chromatin (Beall et al. 2002; Aggarwal and Calvi 2004; Hartl et al. 2007; Vorobyeva et al. 2021). In a stepwise manner, factors recruit and build the replication complex, which is activated by cyclin-dependent kinases, leading to bidirectional fork movement (reviewed by Tower 2004; Claycomb and Orr-Weaver 2005; Nordman and Orr-Weaver 2012). Repeated firing creates an onionskin structure in which peak amplification occurs at origins and DNA levels decrease gradually to a distance of ~ 50 kb in each direction (Park et al. 2007; Kim et al. 2011). The extent of amplification depends on Suppressor of Under-Replication (SuUR), which binds elongating complexes and disrupts their stability, thereby halting fork progression (Nordman et al. 2014).

Transcript abundance is not always correlated with the magnitude of DNA amplification (Kim et al. 2011). Although the expression of the major chorion genes correlates well with the level of DNA amplification (Griffin-Shea et al. 1982), mRNA levels from other genes in these regions vary. Some genes are expressed highly but do not reside in amplified regions, while other genes are amplified but are expressed at a low level (Kim et al. 2011). These inconsistencies may simply reflect the independent evolution of genes in each cluster and the existence of selective pressures on some genes but not others.

Many features of chorion gene amplification are conserved in other species. For example, other Drosophilids employ similar timing, chromatin modifications, and sequence-specific binding complexes to coordinate DNA amplification with eggshell synthesis (Calvi et al. 2007). Studies in the gnat *Sciara coprophila*, which amplifies pupal case genes in an ecdysone-dependent manner (Liew et al. 2013), reveal parallels with *Drosophila* chorion gene amplification, including an association with open chromatin (Lunyak et al. 2002; Urnov et al. 2002) and sequence or structural similarities at origins that allow binding of *Sciara* and *Drosophila* ORC machineries (Bielinsky et al. 2001; Yamamoto et al. 2021). In humans, orthologs of chorion gene amplification factors, such as E2F, are involved in bypassing mechanisms that restrict growth in tumor cells. Moreover, amplification of oncogenes is itself a

Fig. 5. (Continued)
Two kinases, Cyclin-dependent kinase (Cdk2), with its partner Cyclin E, and Dbf4-dependent kinase (Cdc7; Stephenson et al. 2015), activate this prereplication complex. Activation facilitates binding of proliferating cell nuclear antigen (PCNA) and the DNA polymerase complex (Pol), thereby initiating bidirectional fork movement. SUUR limits fork movement to ~50 kb in each direction. Several rounds of reinitiation create branched duplexes such that DNA levels are highest at the origins and gradually decrease on each side (reviewed by Tower 2004; Claycomb and Orr-Weaver 2005; Nordman and Orr-Weaver 2012).

common mechanism to promote tumor progression (Matsui et al. 2013). Thus, analyses of chorion gene amplification reveal fundamental mechanisms of DNA replication and give insight into aberrant processes that impact human health.

Thin eggshell phenotypes help identify new amplification factors

The genes involved in chorion gene amplification are required for DNA replication in other developmental contexts, and as a result, null mutations are lethal. Partial loss-of-function mutations produce phenotypes when protein levels drop below 10% of wild type (Orr et al. 1984; Komitopoulou et al. 1988; Trougakos and Margaritis 1998b), and this observation allowed genetic screens for female sterile mutants that produce eggs with thin eggshells (Underwood et al. 1990; Schüpbach and Wieschaus 1991).

The ease of scoring eggshell defects encouraged investigation of other conserved proteins, to ask if they play a role in chorion gene amplification. For example, the RecQ family helicases are considered "guardians of the genome" (Croteau et al. 2014), and mutations in the human genes cause rare autosomal recessive disorders associated with defective bone growth, premature aging, and cancer (Lu et al. 2014). Genetic analyses of *RecQ4 helicase* mutations suggest that chorion gene amplification requires this protein for initiation but not for elongation (Wu et al. 2008). In contrast, mutations in *mutagen-sensitive 308*, which encodes Polymerase theta, reveal a role for this microhomology-mediated end-joining protein in fork progression (Alexander et al. 2016). The checkpoint protein Claspin is also needed for elongation, while Claspin and Mutagen sensitive 101, a BRCA1 domain-containing protein, are required for initiation as well (Choi et al. 2017).

Terminal patterning and formation of the operculum and micropyle

Patterning of the follicular epithelium along the A/P axis establishes cell types that create specialized eggshell structures (Fig. 6). Formation of the anterior structures, the micropyle, operculum, and collar, involves elaborate cell movements that have merited intensive study (reviewed by Duhart et al. 2017; Horne-Badovinac 2020). Less well characterized is the posterior aeropyle, a raised placode with large pores that facilitate gas exchange. Both eggshell termini are reservoirs for signaling molecules that specify head and tail structures in the embryo. Here, we discuss the patterning, cell migration, and eggshell features that characterize the terminal regions of the egg chamber.

Notch and JAK/STAT signaling define the termini of the egg chamber

Early in egg chamber development (region 2b-S2), after the follicle cells have created a monolayer epithelium around the germ cells, Notch signaling defines 2 unique cells at each pole of the egg chamber (reviewed by Nystul and Spradling 2010). These polar cells facilitate establishment of the stalk cells, which separate individual egg chambers, and they influence the overall architecture of the egg chamber through an unusual, Notch-dependent relay system that helps position the oocyte at the posterior of the egg chamber via differential E-cadherin expression (reviewed by Huynh and Johnston 2004).

Polar cells also influence cell fates within the follicular epithelium by expressing Unpaired (Upd) 1 and Upd3, 2 *Drosophila* cytokines that bind the receptor Domeless to activate Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling in a graded fashion (McGregor et al. 2002; Xi et al. 2003; Wang et al. 2014). Based on expression of *lacZ* reporter constructs,

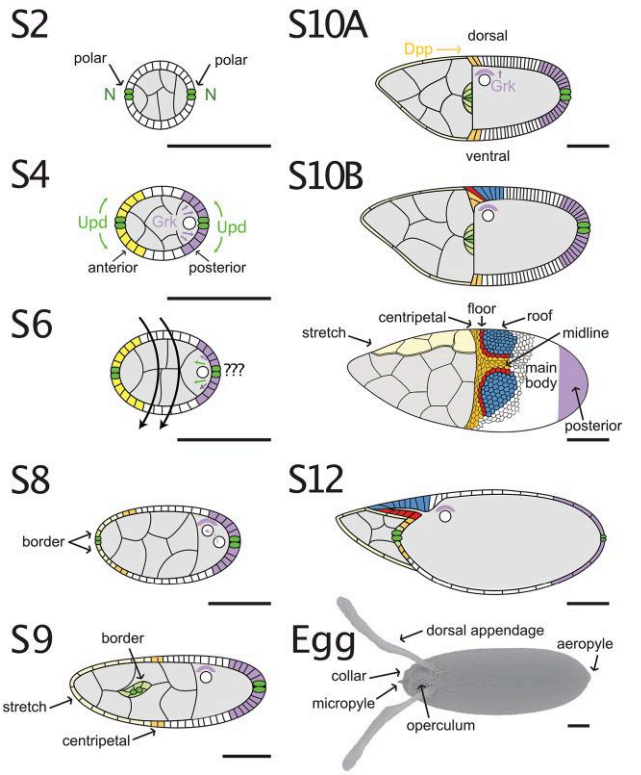

Fig. 6. Patterning and morphogenesis. Representative stages showing progressive patterning and movements of follicle cells. All drawings are lateral cross sections, except the lower S10B example, which is a dorsolateral surface view. By S2, Notch signaling has defined 2 polar cells (dark green) at the anterior and posterior of the egg chamber. During S1–S5, the polar follicle cells secrete the JAK/STAT ligand Upd to pattern terminal regions. At the anterior (yellow), the gradient will specify border cells (light green), squamous stretch cells (yellow), and centripetal cells (orange) (see S8 and S9), while at the posterior, Gurken (Grk, EGF) signaling converts these cells to a posterior fate (purple). At S6, an unknown signal from posterior cells to the oocyte induces a microtubule rearrangement that moves the oocyte nucleus to the anterior. *grk* RNA and protein (crescent) move with the oocyte nucleus. Rotation of the egg chamber by migration of follicle cells on the ECM, which begins slowly at S1 but speeds up at S6, alters egg chamber shape from round to elongated, particularly during S6–S8. At S9, the border cells move between nurse cells toward the oocyte, carrying the polar cells, while the stretch cells flatten. At the transition from S10A to S10B, dorsal anterior follicle cells begin to express markers responding to Grk (EGF) and Dpp (BMP) signals (red, floor cells; blue, roof cells; orange, midline cells). At S10B, the centripetal and midline cells move inward. At S11, the dorsal appendage-forming cells wrap to make 2 tubes while the nurse cells dump their contents into the oocyte, and at S12–S13 the dorsal appendage-forming cells move out over the stretch cells, which envelope the degenerating nurse cells. Scanning electron microscope image of a laid egg (wild-type, Oregon R) reveals structures synthesized by the anterior cells (operculum, micropyle, collar), roof and floor cells (dorsal appendages), main body cells (majority of eggshell), and posterior cells (aeropyle). Image courtesy of Dr. Miriam Osterfield. All black bars = 50 µ.

JAK/STAT signaling defines the termini by S6, but distinct cell type markers do not begin to appear until later in development, at S8 (Grammont and Irvine 2002; Xi et al. 2003). In the anterior of the egg chamber, the Upd signaling gradient defines 3 cell types (Fig. 6): high levels establish a ring of cells surrounding the polar cells called the border cells; moderate levels define the stretch cells, and lower levels determine the centripetal cells. As discussed below, each of these cell types exhibit distinct behaviors that contribute to finishing the egg. At the posterior of the egg chamber, however, Gurken (Grk, EGF) signaling from the oocyte

(Fig. 6; see *Dorsal/ventral patterning and dorsal appendage formation*) converts the posterior terminal domain to a uniquely posterior cell type. Overall, the net effect of JAK/STAT signaling in this context is to distinguish the mid-body follicle cells from those at the termini (reviewed by Duhart et al. 2017; Merkle et al. 2020).

As discussed in the next 2 sections, the border cells and centripetal cells create the anterior face of the eggshell. Although the stretch cells do not secrete eggshell proteins, they do play important structural roles in ensuring the proper morphogenesis of the egg. For example, their affinity for the nurse cells may help position the centripetal cells relative to the oocyte (Weichselberger et al. 2022). The stretch cells provide a substrate for the anterior movement of the dorsal appendage-forming cells (see *Dorsal appendage morphogenesis involves wrapping and tube elongation*; Tran and Berg 2003), and if they fail to engulf the dying nurse cells (see *Nurse cell breakdown*; Timmons et al. 2016), this anterior movement is disrupted (Dorman et al. 2004). The stretch cells also express signals that modulate the shape of the dorsal appendages (Zimmerman et al. 2017; Sustar et al. 2023). Thus, stretch cells contribute to essential functions in finishing the egg.

Border cells migrate collectively and facilitate construction of a sperm entry point

The border cells have emerged as a premier system for studying collective cell migration (reviewed by Montell et al. 2012; Saadin and Starz-Gaiano 2016; Duhart et al. 2017). During S9, 6–10 anterior cells, including the 2 polar cells, delaminate from the follicular epithelium and migrate as a cluster between the nurse cells; by S10, they reach the border with the oocyte, hence their name (King 1970). Analyses of these cells have revealed insights into other types of invasive cell behaviors, including tumor cell metastasis (reviewed by Cai and Montell 2014; Rosales-Nieves and González-Reyes 2014; Cheung and Ewald 2016). Here, we summarize the key features of this model system.

In addition to Notch and JAK/STAT signaling as discussed above, the Hippo pathway facilitates cell fate specification of the border cells by regulating polar cell expression of Upd (Lin et al. 2014). Activation of STAT by Upd induces the expression of and modulates the activity of Slbo, a C/EBP transcription factor encoded by *slow border cells* (Montell et al. 1992). Precise levels of Slbo and JAK/STAT signaling are important both for defining the correct number of border cells and for controlling their migration. As a result, the gene regulatory network has evolved positive and negative feedback mechanisms, including input from other growth control pathways, to maintain proper regulation of these factors (reviewed by Saadin and Starz-Gaiano 2016; Kang et al. 2018; Sharma et al. 2018; Ogienko et al. 2020).

Mosaic analyses demonstrate that delamination of the border cells at S9 depends on local production of the steroid hormone ecdysone in the follicle cells (Domanitskaya et al. 2014). Steroid receptor activation (Bai et al. 2000; Cherbas et al. 2003) regulates target genes (Manning et al. 2017) specifically in border cells through JAK/STAT-mediated downregulation of Abrupt, a negative regulator of the steroid receptor coactivator Taiman (Jang et al. 2009; reviewed by Bellés and Piulachs 2015). One important outcome of this hormonal regulation is to modulate E-cadherin such that the central polar cells maintain high levels, the outer border cells express modest levels, and nurse cells express the lowest level (Hackney et al. 2007; Graeve et al. 2012). These differences in adhesive properties facilitate movement between the nurse cells toward the oocyte (Cai et al. 2014). High E-cadherin levels also prevent formation of a tubular lumen during cell migration (Wang, Wang, Liu et al. 2020).

As the border cells dissociate from their sister follicle cells, i.e. the cells that will become the stretch cells, their movement toward the posterior coincides with the flattening of the stretch cells into a squamous epithelium (Kolahi et al. 2009; reviewed by Duhart et al. 2017). Although the mechanism that achieves this coordination is not clear, it is known that stretch cell flattening is regulated by BMP, Hippo, and ecdysone signaling (Brigaud et al. 2015; Fletcher et al. 2018; Borreguero-Muñoz et al. 2019; Jia et al. 2022).

In addition, border cells maintain an epithelial architecture when moving between nurse cells (Niewiadomska et al. 1999). The cluster has a leader cell that extends protrusions between the nurse cells, follower cells that surround and transport the 2 central polar cells, and apicobasal polarity as revealed by differential E-cadherin localization (Montell et al. 2012). The border cells communicate with each other and with the polar cells through planar cell polarity (PCP), Jun-kinase, and G protein-coupled receptor signaling (Bastock and Strutt 2007; Llense and Martín-Blanco 2008; Anllo and Schüpbach 2016). These signals regulate cell polarity proteins, motor proteins, and adhesion molecules, thereby ensuring epithelial cohesiveness during delamination and migration (reviewed by Montell et al. 2012; Saadin and Starz-Gaiano 2016; Aranjuez et al. 2016; Cha et al. 2017; Chen, Kotian, et al. 2019; Mishra et al. 2019; Kotian et al. 2021; Campanale et al. 2022). Live image analyses show that the initial phase of migration is rapid and depends heavily on maintenance of cluster architecture. Nevertheless, the border cells do alter their position in the cluster. In the later, slower phase, this reorganization is more dramatic, and the entire assemblage tumbles as it moves to the posterior (Bianco et al. 2007; Prasad and Montell 2007; reviewed by Sano et al. 2007; Cliffe et al. 2017).

The cell located at the leading edge of the cluster exhibits protrusive behavior, which depends on tight regulation of actin and myosin (reviewed by Montell et al. 2012; Saadin and Starz-Gaiano 2016; Roberto and Emery 2021). Elegant in vivo studies using light-activated Rac, a GTPase that regulates the actin cytoskeleton, demonstrate that directionality and speed of movement depend on the location and strength of Rac activity (Wang et al. 2010). Membrane trafficking contributes to this regulation by ensuring that activated Rac remains localized to the leading edge where other GTPase regulators control its ability to induce protrusive behavior (Fernández-Espartero et al. 2013; Ramel et al. 2013; Chang et al. 2018). Follower cells also require Rac to modulate actin dynamics, maintain cluster cohesion, and promote crawling (Campanale et al. 2022). During migration, 3 distinct polarity complexes coordinate the behavior of cells within the cluster through localization of atypical protein kinase C (aPKC) and a guanine nucleotide exchange factor, chondrocyte-derived ezrin-like domain-containing protein (Cdep) (Wang et al. 2018; Campanale et al. 2022). Additional in vivo studies using an E-cadherin tension sensor provide unparalleled views into how cells perceive signals and coordinate their movements (Cai et al. 2014). These and other powerful genetic and imaging tools have allowed detailed characterizations of the regulatory pathways that control cellular dynamics at the leading and trailing edges of the cluster (Plutoni et al. 2019; Wang, Guo et al. 2020; Fox et al. 2020; Badmos et al. 2021; Lamb et al. 2021).

The border cells delaminate from the follicular epithelium and migrate medially, in between the nurse cells, from the anterior toward the posterior. To identify the signals that provide spatial direction to the border cells, an overexpression screen of random genes in the genome found 2 signaling molecules that could confuse border cells during their migration. These ligands were Vein (1 of 4 fly EGF homologs) and PDGF- and VEGF-related factor 1

(Pvf1, which is related to vertebrate Platelet-derived growth factor, PDGF, and Vascular endothelial growth factor, VEGF; Duchek et al. 2001). This gain-of-function approach was instrumental in identifying these guidance cues since loss of either molecule alone produced only minor delays in border cell movement. Other approaches clarified the process in part by developing tools to locally misexpress ligands and thereby redirect border cells to new sites (McDonald et al. 2003, 2006). These studies showed that 2 other EGF ligands, Keren and Spitz, together with Pvf1, guide the border cells to the oocyte. The tumor suppressor gene *abnormal wing discs* (human *Nme1/Nm23*) must be downregulated to optimize integration of these guidance cues with JAK/STAT signaling and allow forward movement (Nallamothu et al. 2008). In addition to these chemical cues, the border cells interpret the cellular geometry and packing of the nurse cells to choose a path of least resistance as they move forward (Mekus et al. 2018; Peercy and Starz-Gaiano 2020; Dai et al. 2020).

When the border cells arrive at the oocyte at S10A, they move dorsally under the influence of Grk signaling (Duchek and Rørth 2001). This movement is transient, however, since by S11 the cells occupy a ventral position at the anterior of the egg. There they establish new contacts with the oocyte in an innexin- and MT-dependent manner (Miao et al. 2020) and then collaborate with centripetally migrating cells to generate the micropyle (see *Centripetal and midline cells migrate inward to create the operculum and micropyle*; reviewed by Horne-Badovinac 2020). Electron and light microscopy studies show that the polar cells extend MT- and actin-rich processes toward the oocyte; these processes embed into the vitelline membrane and block coalescence of chorion proteins secreted by neighboring cells (Zarani and Margaritis 1986; Edwards et al. 1997). As a result, the micropyle is a small cone-shaped structure, 20 μM long and 10 μM in diameter at its base, with a central hole for sperm entry leading to a specialized vitelline membrane that must be penetrated during fertilization (Turner and Mahowald 1976). Delay or failure of border cell migration results in a micropyle that lacks this hole, and thus, border cell mutants are sterile (Montell et al. 1992).

Centripetal and midline cells migrate inward and create the operculum, collar, and micropyle

Two populations of follicle cells, the centripetal cells and the midline cells, cooperate to synthesize several eggshell structures: the anterior face of the eggshell or operculum, a surrounding collar, and the micropyle (reviewed by Duhart et al. 2017). These cells are defined in part by the initial gradient of Upd that distinguishes main body cells from termini (Xi et al. 2003; see also *Notch and JAK/STAT signaling define the termini of the egg chamber*); 2 other pathways, Decapentaplegic (Dpp, BMP) and Gurken (Grk, EGF), subsequently contribute to their specification (Dobens et al. 2000; Yakoby, Lembong et al. 2008; see also *Gurken is a morphogen that cooperates with other signals to specify fates*). High levels of these latter signals define a 2-row ring of cells at the anterior-most region of columnar follicle cells (Dobens et al. 2005; Chen and Schüpbach 2006; Shravage et al. 2007; Charbonnier et al. 2015) as well as a thin stripe of cells on the dorsal midline that separates the 2 dorsal appendage-forming primordia (Lachance et al. 2009; Zartman, Kanodia, Cheung et al. 2009). One key feature of this process is the downregulation of Bunched, a member of the TSC22 family of leucine zipper transcription factors (Dobens et al. 2000). Bunched acts in a negative feedback loop with the C/EBP ortholog Slbo to maintain distinct cell fates within the epithelium; this interaction resembles a regulatory network that controls fat cell differentiation in mammals (Levine et al. 2007).

Centripetal migration is a 2-phase process (Parsons et al. 2023). Beginning at S10B with cells on the dorsal side of the egg chamber, leading centripetal cells invade the space between the nurse cells and oocyte (King 1970). One hypothesis suggests that by localizing actin and myosin at their apical (inward facing) surfaces, these cells create a purse string structure that contracts to drive the movement of the cells toward the interior of the egg chamber until the entire anterior face of the oocyte is covered (Edwards and Kiehart 1996). Consistent with this hypothesis, capping protein beta is required for centripetal migration (Ogienko et al. 2013). Based on the behaviors of the centripetal cells and on studies of actin dynamics in the border cells or in the embryo, this actomyosin complex could also be involved in remodeling of centripetal cell shapes (e.g. apical constriction of the leading cells), generating the cortical tension needed to squeeze between the germ cells, or producing filopodia or lamellipodia (Aranjuez et al. 2016; Kiehart et al. 2017; Parsons et al. 2023).

In the second phase of centripetal migration, more posterior following cells change their orientation as they tilt downward to invade, but their aspect ratio does not change (Parsons et al. 2023). During ingression, ~2 squamous cells move inward with the centripetal cells (Tran and Berg 2003; Parsons et al. 2023); the function of these accompanying cells is not known. At the basal surface, both the centripetal cells and the midline cells constrict, and these cells exchange edges with lateral neighbors to allow their reorganization and to dissociate from the ECM, the dorsal appendage-forming floor cells, and other main-body follicle cells (Levine et al. 2010; Osterfield et al. 2013; Parsons et al. 2023). These studies demonstrate that centripetal migration in *D. melanogaster* is actively invasive, unlike the process in some other insects, which lack centripetal migration or use a simple folding mechanism to achieve ingression (reviewed by Jaglarz et al. 2008, 2010; Garbiec and Kubrakiewicz 2012; Garbiec et al. 2016).

Several types of cell adhesion molecules play key roles in mediating centripetal migration. The homophilic cell adhesion molecule E-cadherin (encoded by *shotgun*) is required in both the germline and soma for inward movement (Niewiadomska et al. 1999; Parsons et al. 2023), while *18-wheeler*, which encodes a Toll-like receptor, is needed in centripetal and midline cells (Kleve et al. 2006). Other cell adhesion molecules, such as Fasciclin 3 (Ward and Berg 2005; Shravage et al. 2007) and Cadherin 99C (D'Alterio et al. 2005; Zartman, Kanodia, Yakoby et al. 2009), are also expressed in these cells and likely mediate their activities.

The onset of centripetal migration marks the decision by the egg chamber to complete oogenesis. Prior to this time, egg chambers maturing in culture demand nutritional inputs and growth factors (e.g. insulin and fetal bovine serum) for their continued development. At S10B, however, egg chambers will mature to S14 in a chemically defined medium (Robb's R14 medium; reviewed by Peters and Berg 2016a).

Like other changes that occur at this time (see *Eggshell composition* and *Chorion gene amplification*), centripetal migration begins following a pulse of ecdysone signaling (Hackney et al. 2007). BMP signaling may also be required since ligand, receptors, and transcription factors are all expressed highly in these cells (Twombly et al. 1996; Jékely and Rørth 2003; Shravage et al. 2007; Yakoby, Lembong et al. 2008). Distinguishing between a role for BMP in cell fate specification and morphogenesis is difficult, however, since no alleles or constructs exist that disrupt the function only during migration.

In addition to these temporal cues, the spatial positioning of the centripetal cells relative to the oocyte might impact their

ability to migrate (Schüpbach and Wieschaus 1991; Mahone et al. 1995; Swan and Suter 1996; Keyes and Spradling 1997). For example, in mutants that produce open-ended eggshells, such as *chalice* or *cup*, oocyte growth is retarded relative to the maturation of the follicular epithelium. As a result, the centripetal cells reside too far to the anterior, and they either fail to initiate inward movement or they migrate aberrantly between nurse cells.

At the transcriptional level, the C/EBP homolog Slbo is highly expressed in centripetal cells, and although not essential for centripetal migration (Montell et al. 1992), it coordinates cell movements with nurse cell dumping by regulating expression of E-cadherin and other surface proteins (Levine et al. 2007). Transcriptional profiling of Slbo-expressing cells (border cells and centripetal cells) (Wang et al. 2006) and comparisons of wild-type egg chambers with those in which EGF or BMP signaling is manipulated uncover additional genes with intriguing expression patterns and potential roles in centripetal and midline cell migration (Yakoby, Bristow et al. 2008).

Scanning and transmission electron microscopy reveal regional differences in operculum structure (Turner and Mahowald 1976; Margaritis et al. 1980), presumably due to the input of the 3 cell types that affect its assembly (border cells, centripetal cells, and midline cells). The size and shape of follicle cell imprints differ: ventrally, surrounding the micropyle, they are small hexagons, while dorsally they are larger, more elongated rhomboids. Because egg chambers that lack border cells still produce a micropyle, albeit misshapen and lacking a sperm entry hole (Montell et al. 1992), the assumption is that the centripetal cells are the major source of vitelline membrane and chorion proteins that make up that structure. The most specialized region is the collar, which exhibits a patchy vitelline membrane covered by 2 separate layers of endochorion, so constructed to allow ready disintegration upon larval hatching (Margaritis et al. 1980). As discussed above, defects in centripetal cell migration lead to open-ended eggshells, and females harboring mutations affecting this process are sterile (Schüpbach and Wieschaus 1991; Twombly et al. 1996; Edwards and Kiehart 1996).

Terminal follicle cells initiate head and tail formation in the embryo

Terminal patterning of the egg chamber is important not only for creating specialized structures of the eggshell but also for establishing the unsegmented terminal regions of the embryo. This process exhibits many parallels with D/V patterning of the embryo (see *Gurken is a morphogen that cooperates with other signals to specify fates*) and indeed shares some genes. The downstream components of the pathway are well known and constitute a premier system for exploring quantitative behavior of mitogen activated protein kinase (MAPK) signaling (Goyal et al. 2018; Smits and Shvartsman 2020).

Briefly, Trunk, a cysteine knot protein related to vertebrate Noggin (Duncan et al. 2013), activates a uniformly distributed receptor tyrosine kinase, Torso, only at the embryo poles, as discussed below. Signal transduction through Ras, Raf, MEK, and MAPK leads to regional degradation of the transcriptional repressor Capicua (Catalan for "head and tail"), thereby allowing expression of the zygotic genes *tailless* and *huckebein* (reviewed by Li 2005; Mineo, Furriols et al. 2018). Intriguingly, cell cycle-dependent degradation of Torso protects the germ cells, which reside at the posterior pole within the domain of activated Torso, from being specified as somatic terminal cells (Pae et al. 2017; Colonnetta et al. 2021).

Through numerous challenging experiments, the upstream mechanisms of the Torso pathway are becoming clear. Transcripts encoding the Trunk ligand are loaded maternally into the embryo, but the protein is cleaved and secreted only at the termini (Casali and Casanova 2001; Henstridge et al. 2014; Johnson et al. 2015). This localized activation of the pathway occurs through the earlier production of Torso-like by small groups of terminal follicle cells (Stevens et al. 1990; Savant-Bhonsale and Montell 1993; Martin et al. 1994). Torso-like, which is related to perforin proteins that poke holes in membranes (Johnson, Henstridge et al. 2017), is secreted into the vitelline membrane (Stevens et al. 2003) and is stored there until egg activation, when it translocates to the embryo plasma membrane (Mineo et al. 2015). Cell culture experiments suggest that Torso-like facilitates activation of Torso by mediating or stabilizing dimerization of the receptor upon binding to its ligand (Amarnath et al. 2017). How a perforin-like protein might facilitate this interaction is unclear, although one possibility is that it alters the structure of the plasma membrane near Torso (reviewed by Santos et al. 2015). Consistent with this hypothesis, physical perturbation of the membrane can mimic Torso-like activity (Mineo, Fuentes et al. 2018), and a screen for new components of the Torso pathway identified Tetraspanin 3A, which might also act by altering local membrane structure (Johns et al. 2018).

Mechanistically, the localized activation of the Torso signaling pathway by an eggshell-associated protein, Torso-like, resembles the process that establishes D/V patterning in the embryo (see *Gurken is a morphogen that cooperates with other signals to specify fates*; reviewed by Stein and Stevens 2014; Merkle et al. 2020). Both pathways use autocrine signaling mechanisms, and Trunk, the ligand for Torso, shares structural features with Spätzle, the ligand for Toll (Morisato and Anderson 1994; Casanova et al. 1995). Both pathways require the activities of Polehole, Nasrat, and Closca, which are made in the oocyte and secreted into the vitelline membrane (Jiménez et al. 2002; Ventura et al. 2010). There they facilitate late steps in the processing of Nudel protein, a component of the protease cascade that activates Spätzle, and they stabilize Torso-like at the embryo poles (Mineo et al. 2017). A recent study also shows that Torso-like activity at the poles coordinates cell shape changes during ventral furrow formation (Johnson, Moore et al. 2017), a morphogenetic process induced by D/V patterning.

These striking features of *Drosophila* terminal patterning involve the likely co-option of Torso signaling from a molting pathway and are recent evolutionary innovations (reviewed by Duncan et al. 2013; Weisbrod et al. 2013; Auman and Chipman 2017; Skelly et al. 2019; Taylor et al. 2019).

Dorsal/ventral patterning and dorsal appendage formation

The dorsal appendages are the most prominent eggshell specializations. Studies on these structures led to the exciting discovery that *gurken* (*grk*) (German for "cucumber") regulates D/V patterning of both the eggshell and embryo (Schüpbach 1987). *grk* encodes a homolog of vertebrate EGF (Neuman-Silberberg and Schüpbach 1993) and activates the EGF receptor (EGFR; encoded by *torpedo*; Price et al. 1989; Schejter and Shilo 1989). Subsequent screens identified additional components of the EGF signaling pathway and other factors that regulate *grk* in some way (reviewed by Berg 2005; Schüpbach 2016, 2019; Merkle et al. 2020).

In the next sections, we summarize the essential steps associated with D/V axis formation and dorsal appendage morphogenesis, and we show how Grk signaling also regulates A/P patterning. We direct the reader to timely reviews for more in-depth

discussions of the key findings (Roth 2003; Horne-Badovinac and Bilder 2005; Stein and Stevens 2014; Osterfield et al. 2017; Merkle et al. 2020).

Gurken (EGF) signaling creates asymmetry

Although D/V patterning mutants such as *grk* exhibit striking changes in dorsal eggshell structures, strong loss-of-function mutations also generate eggs with 2 anterior ends, i.e. the posterior consists of an operculum, collar, and micropyle rather than an aeropyle (Schüpbach 1987). These phenotypes showed that EGF signaling regulates both D/V and A/P patterning of the egg chamber. Subsequent molecular studies showed that *grk* mRNA and Grk protein are first localized to the posterior of the egg chamber and then move to the outer anterior cortex of the oocyte, and this shift in subcellular localization regulates distinct signaling processes (Neuman-Silberberg and Schüpbach 1993). The current hypothesis is that early Grk signaling to posterior follicle cells at S1–S5 establishes posterior cell fates. These cells then signal back to the oocyte at S6, inducing a relocalization of *grk* products to the anterior, where subsequent signaling at S10 establishes dorsal follicle cell fates (González-Reyes et al. 1995; Roth et al. 1995; González-Reyes and Johnston 1998; reviewed by Merkle et al. 2020).

Two other findings support this hypothesis. First, MTs undergo a major reorganization during S6–S8 (Theurkauf et al. 1992; Gillespie and Berg 1995; Pokrywka and Stephenson 1995). This reorganization repositions the plus ends of MTs at the posterior of the oocyte and the minus ends at the anterior, pushing the oocyte nucleus to a random position at the anterior of the oocyte and relocalizing *grk* transcripts and protein in the process (reviewed by Bernard et al. 2017). Importantly, other embryonic patterning molecules, e.g. those encoded by *bicoid* and *oskar*, localize to the anterior or posterior of the oocyte due to their dependence on different classes of MT motor proteins (reviewed by Merkle et al. 2020).

The second finding was that Notch is required in the follicle cells for proper localization of patterning molecules in the oocyte (Ruohola et al. 1991). We now know that Notch signaling is required to differentiate terminal follicle cells (see *Notch and JAK/STAT signaling define the termini of the egg chamber*) and thus works in conjunction with EGF signaling to establish posterior follicle cell fates.

Since these initial discoveries, several groups have set out to identify the signal sent from posterior follicle cells back to the oocyte (Deng and Ruohola-Baker 2000; MacDougall et al. 2001; Deng et al. 2003; Poulton and Deng 2006; Meignin et al. 2007; Polesello and Tapon 2007; Yu et al. 2008; Sun et al. 2011; Wittes and Schüpbach 2018; and others cited in Merkle et al. 2020). The assumption is that the molecule(s) and its pathway are involved in other biological processes, and therefore, the screening strategies have exploited methods to bypass earlier requirements in development. Although these studies revealed contributing factors, the signaling mechanism that initiates the dramatic cytoskeletal changes in the oocyte is still unknown. One unexplored possibility, hinted at by studies on the maintenance of oocyte polarity in S10 egg chambers (Milas et al. 2022), is that mechanical contact between posterior follicle cells and the oocyte might facilitate this critical reorganization of the oocyte MT network.

Gurken is highly regulated

Since Grk signaling initiates patterning of the entire body plan, it is under tight control (reviewed by Johnstone and Lasko 2001; Kugler and Lasko 2009; Lasko 2012; Derrick and Weil 2017). In fact, Grk is so important that hundreds of genes impinge on its function, including inputs from growth regulatory pathways and meiotic checkpoints (reviewed by Merkle et al. 2020).

Mosaic studies reveal that *grk* mRNA is made in the nurse cells, packaged into RNP particles, and transported rapidly into the oocyte via dynein motors (Duncan and Warrior 2002; MacDougall et al. 2003; Cáceres and Nilson 2005; Rom et al. 2007). During transport, limiting amounts of the poly-A binding protein Orb (and therefore the poly-A polymerase Wispy) ensure that *grk* transcripts maintain short poly-A tails, repressing translation until the RNA is localized properly in the oocyte (Tan et al. 2001; Wong et al. 2011; Norvell et al. 2015; Davidson et al. 2016). Translational repressors (e.g. Squid; Cáceres and Nilson 2009) contribute to this restricted expression.

Localization of *grk* RNA within the oocyte requires the activity of over a dozen of RNA binding proteins or MT-associated proteins. Unlike most other localized transcripts in which sequences in the 3′ UTR are sufficient to target the mRNA to a locale, *grk* transcripts contain regions in the 5′ UTR and coding sequence that also contribute to localization (Bor et al. 2005; Thio et al. 2000; Lan et al. 2010).

Once *grk* transcripts are localized to the dorsal anterior corner of the oocyte, Squid facilitates a switch in the behavior of the transport particles; this switch creates static ribonucleoprotein particles called "sponge bodies" that anchor *grk* message and prevent its diffusion throughout the oocyte (Delanoue et al. 2007). Within these endoplasmic reticulum-associated sponge bodies, translational repressors are displaced, and the DEAD box helicase Vasa facilitates translation (reviewed by Lasko 2012). Subsequent Grk secretion requires cleavage by the intramembrane protease Rhomboid (Strisovsky et al. 2009). Once secreted into the extracellular space between the oocyte and follicle cells, glycosphingolipids present on the outer leaflet of the oocyte plasma membrane modulate Grk diffusion and thereby shape the gradient of Grk protein (Pizette et al. 2009).

Grk's key role in fly development is underscored by the fact that production of the protein depends on the integrity of other critical processes in the egg chamber. For example, meiotic recombination facilitates DNA exchange and segregation of homologs during oocyte development (reviewed by Lake and Hawley 2012), but a failure to repair the double-strand breaks that mediate recombination elicits a checkpoint response that blocks translation of *grk* RNA into protein (reviewed by Merkle et al. 2020). Similarly, mutations that disrupt production of Piwi-interacting RNAs (piRNAs, also called repeat associated small interfering RNAs or rasiRNAs) allow expression and mobilization of transposable elements, inducing double-strand breaks and activating checkpoints that regulate the amount of Grk protein (reviewed by Saito 2013; Hsu et al. 2020). Grk levels can also be affected by competition for RNA-binding or transport proteins; for example, retrotransposons such as *I* factor, *G2*, and *Jockey* carry cis elements that produce secondary structures mimicking *grk* transcripts and interfering with *grk* mRNA regulation (Bor et al. 2005; Hamilton et al. 2009). In general, when the oocyte suffers irreparable DNA damage, egg chamber maturation continues, but the *grk* "quality-control checkpoint" prevents transmission of that mutant DNA into progeny by inhibiting axis specification and thereby disrupting embryonic development. Thus, *grk* is a hub that integrates information from numerous processes to ensure robust gamete production.

In contrast, if oocyte development is normal but female flies suddenly face a reduction in nutritional resources, a bypass mechanism allows synthesis of Grk protein (Ferguson et al. 2012) even though most other transcripts experience a block in

translation initiation (Richter and Sonenberg 2005; reviewed by Jackson et al. 2010). This response may increase survival of the resulting progeny (Burn et al. 2015).

Gurken is a morphogen that cooperates with other signals to specify fates

In early stages of oogenesis, low levels of Grk specify posterior follicle cell fates, while at later stages, moderate or high levels of signaling specify dorsal appendage- or operculum-forming cell fates, respectively. Signaling levels that lie below a minimum threshold result in cells with a "default" main body or ventral fate. Although in each context signaling occurs through the Ras/Raf/MAPK pathway (reviewed by Berg 2005), the concentration of Grk ligand determines the outcome (Goentoro et al. 2006; Chang et al. 2008; Lachance et al. 2009; Zartman, Kanodia, Cheung et al. 2009; Wang and Pai 2011).

Two other pathways function with *grk* in follicle cell specification. In posterior follicle cells, JAK/STAT signaling (see *Notch and JAK/STAT signaling define the termini of the egg chamber*) facilitates expression of 2 paralogous T-box transcription factors, Midline and H15, in response to early Grk signals, and these factors then prevent posterior follicle cells from responding to later higher levels of EGF signaling (Lomas et al. 2013, 2016). In the anterior, graded levels of Dpp (BMP) signaling from stretch follicle cells combine with Grk signaling to define unique cell types along the anterior-posterior axis (Twombly et al. 1996; Deng and Bownes 1997; reviewed by Dobens and Raftery 2000; Dobens et al. 2000; Peri and Roth 2000; Chen and Schüpbach 2006; Shravage et al. 2007; Yakoby, Lembong et al. 2008; Charbonnier et al. 2015; Duhart et al. 2017). Together these pathways create diverse patterns of gene expression (Yakoby, Bristow et al. 2008; Zartman, Kanodia, Yakoby et al. 2009; Cheung et al. 2011). Thus, the follicle cells integrate information from multiple signaling pathways to specify subpopulations that create distinct eggshell structures, including dorsal appendage-forming cells marked by expression of the BTB zinc finger transcription factors encoded by *broad* (reviewed by Duhart et al. 2017; Osterfield et al. 2017).

Several mathematical models have reshaped our interpretation of the underlying molecular mechanisms by quantifying the initial Grk and Dpp gradients (Goentoro et al. 2006; Lembong et al. 2008). Other models show how those gradients feed into gene regulatory networks (Lembong et al. 2009; Zartman, Kanodia, Cheung et al. 2009) that specify subpopulations within the epithelium (Simakov et al. 2012; Fauré et al. 2014). These approaches provide predictive models to explain alternative patterning processes in other species (Zartman et al. 2011).

One key function of Grk signaling is to regulate D/V patterning in the embryo. More than a dozen genes are required for the downstream steps, but the upstream initiating event occurs when Grk-activated EGFR causes dorsal follicle-cell repression of *pipe*, which encodes 10 related glycosaminoglycan-modifying enzymes (Sen et al. 1998; Sergeev et al. 2001). Pipe activity in ventral follicle cells modifies components of the vitelline membrane (Zhang, Stevens et al. 2009; Zhang, Zhu et al. 2009), creating a region of sulfated eggshell that triggers localized cleavage of the ligand Spätzle, which was made in the embryo and secreted uniformly into the perivitelline space. Cleaved Spätzle activates the Toll signaling pathway, leading to translocation of the Dorsal transcription factor (a homologue of vertebrate Nuclear factor kappa-light-chain-enhancer of activated B cells, NF-κB) into nuclei on the ventral side of the embryo (reviewed by Stein and Stevens 2014; Schloop et al. 2020; Merkle et al. 2020). Intriguingly, the Toll pathway is quite ancient and regulates innate immunity in all metazoans (Lemaitre et al. 1996; Kimbrell and Beutler 2001). Through evolutionary mechanisms that are not yet clear, arthropods have co-opted this pathway to establish D/V polarity (Lynch and Roth 2011; Vreede et al. 2013; Bressan and Araujo 2021).

Dorsal appendage morphogenesis involves wrapping and tube elongation

The 2 dorsal appendages of the eggshell consist of cross-linked chorion protein complexes interspersed with air-filled "plastrons" that facilitate gas exchange for the embryo (Hinton 1960). They are made by 2 patches of follicle cells that create elongated tubes, secrete eggshell proteins into the lumens of the tubes, and then slough off during ovulation. Thus, the dorsal appendages on the laid egg serve as readout for proper tube formation, similar to the way that a Jell-o's shape reveals its mold. In *D. melanogaster*, these respiratory structures resemble oars, with a long, rounded stalk and a flat paddle, but the number and shape of appendages vary widely among Drosophilids, offering an outstanding system for comparative studies (Throckmorton 1962; reviewed by Osterfield et al. 2017).

Analysis of markers in fixed tissues (Dorman et al. 2004; Ward and Berg 2005; Osterfield et al. 2013) and live imaging of egg chambers developing in culture (Dorman et al. 2004; Osterfield et al. 2013; reviewed by Peters and Berg 2016a) demonstrate that the 2 patches of cells specified by EGF and BMP signaling first "wrap" to make a cone-shaped structure parallel to the follicular epithelium sheet. Wrapping is one of a few highly conserved tube-forming mechanisms and produces the neural tube in most vertebrates, the ventral furrow during gastrulation of the fly embryo, and other foundational rudiments during organ formation (reviewed by Hogan and Kolodziej 2002; Lubarsky and Krasnow 2003; Andrew and Ewald 2010). Wrapping during dorsal appendage tube formation involves distinct behaviors by 2 subpopulations in each patch of cells: the roof cells constrict their apical surfaces (which face the oocyte), bending the epithelium up and out of the flat plane (Dorman et al. 2004), and the floor cells dive beneath the roof cells, exchanging neighbors to zip up and seal the floor (Dorman et al. 2004; Osterfield et al. 2013). The mechanical forces that drive these movements likely arise from myosin-based apical tension. Mathematical vertex models support this hypothesis and suggest that forces within the floor cells are particularly important in creating the tube (Osterfield et al. 2013).

In the next phase of dorsal appendage formation, lateral roof cells move toward the dorsal midline; this intercalation narrows and lengthens the tubes (Dorman et al. 2004; Ward and Berg 2005). The roof cells expand their apical surfaces in a biased fashion (Peters and Berg 2016b; Espinoza and Berg 2020), and the basal surfaces of both roof and floor cells extend filopodia and lamellapodia while crawling anteriorly (Dorman et al. 2004; Ward and Berg 2005; Boyle and Berg 2009). Continued cell shape changes and rearrangements create the rounded stalk and flattened paddle that exemplify the dorsal appendages of this species (reviewed by Osterfield et al. 2017).

Mosaic analyses and laser ablation studies demonstrate that signals from within and outside the tubes regulate tube shape. These studies show that the floor cells and first row of roof cells control tube elongation (Boyle and Berg 2009; Boyle et al. 2010), while signals from the nurse cells and stretch follicle cells control tube closure and tube shape (Rittenhouse and Berg 1995; Tran and Berg 2003). Proteomic analyses of purified stretch cells identified a novel class of growth factors, the Imaginal disc growth factors, that when overexpressed induce dramatic changes in cell shape and cell adhesion, creating short, broad, open tubes

(Zimmerman et al. 2017). In contrast, the complete loss of all 6 family members (the first example of gene editing of a dispersed gene family) reveals a requirement for these factors in maintaining dorsal appendage tube architecture. The entire epithelium is disrupted, particularly in response to a brief pulse of CO_2 (Sustar et al. 2023). How these factors modulate tube behaviors is not yet understood.

Tube formation in other species resembles that in *D. melanogaster* with 2 striking exceptions. First, in *D. willistoni*, enhanced Grk signaling creates a dorsal ridge in addition to the 2 appendages. Amazingly, introduction of *D. willistoni grk* into *D. melanogaster* is sufficient to create a dorsal ridge (Niepielko and Yakoby 2014). The second exception occurs in *Scaptodrosophila*; there, females produce eggs with a variable number of long, thin dorsal appendages, and they do so by skipping the wrapping phase and simply elongating pairs of floor cells, which pull roof cells behind them. Several factors hint at possible molecular mechanisms for this remarkable divergence in tube formation, including differences in the localization patterns of myosin and the apical basal polarity protein Bazooka (Par3) (Osterfield et al. 2015), and a heterochronic shift in the transcriptional regulatory pathway governing tube elongation (O'Hanlon et al. 2018).

Thus, dorsal appendage formation provides a relatively simple model to explore the processes that regulate the mechanisms and evolution of tube formation.

Mechanism of egg elongation

S1 egg chambers are round, but the finished egg is elongated along the anterior-posterior axis. Early analyses of mutants that fail to elongate, such as *kugelei* (German for ball egg) and *bola* (Portuguese for ball), demonstrated that interactions between basal cytoskeletal networks and the ECM are required for egg morphogenesis (Gutzeit et al. 1991; Bateman et al. 2001; Frydman and Spradling 2001). Later live imaging studies revealed roles for pulsed actin-based contractions and a physical rotation of the egg chamber along the A/P axis, implicating mechanical forces in the process (He et al. 2010; Haigo and Bilder 2011; reviewed by Gates 2012; Isabella and Horne-Badovinac 2015a; Cetera and Horne-Badovinac 2015). Here, we describe how egg chamber elongation offers a simple system to study the biophysical and cell biological aspects of cellular morphogenesis.

Actin-based contractions and collective cell migration shape the egg chamber

Egg chamber elongation involves at least 4 distinct follicle cell behaviors (Fig. 7): pulsed apical constrictions at the poles of the egg chamber; collective cell migration that rotates the entire egg chamber around the A/P axis; pulsed basal contractions centered in the middle of the egg chamber; and follicle cell–matrix interactions that sustain the elongation process. These behaviors create mechanical forces that drive egg shape changes during oogenesis.

The first step in egg chamber elongation involves the establishment of a gradient of JAK/STAT signaling radiating from the 2 poles of the egg chamber (see *Notch and JAK/STAT signaling define the termini of the egg chamber*; reviewed by Horne-Badovinac and Bilder 2005). During S3–S7, this gradient induces pulses of apical constriction that pull on the 2 ends of the egg chamber and, due to the nonelastic nature of the tissue, slowly cause the sphere to elongate. Although myosin II is localized apically and is required for pulsatile contractions, the JAK/STAT pathway does not regulate myosin-II activity directly. Furthermore, cells intercalate but do not exhibit a clear planar cell polarity (Alégot et al. 2018). At the same time, JAK/STAT signaling induces expression

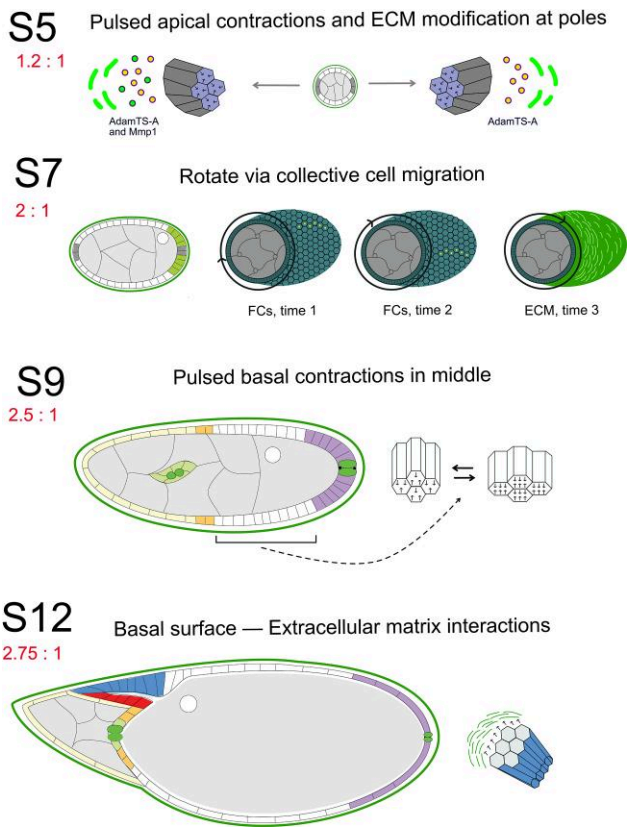

Fig. 7. Mechanisms of egg elongation. The round egg chamber elongates through distinct follicle cell behaviors. During S3–S7 (represented by the S5 egg chamber), a gradient of JAK/STAT signaling at the poles induces apical constriction of the follicle cells, pulling the germ cells outward. JAK/STAT signaling also induces expression of proteases (AdamTS-A and MMP1) that modify the ECM (outer, thick, light green lines). As shown by the S7 egg chamber, the main force driving elongation is the rotation of the egg chamber along its long axis. The follicle cells (middle two egg chambers, dark green) walk in the same direction; yellow dots mark the position of a row of cells at different times during development. The cells move slowly at first (S1–S5), but the speed picks up during S6–S8. During their migration, the follicle cells secrete ECM proteins into the basement membrane (far right egg chamber, light green) in a graded fashion such that the polar regions exert less tension on the egg chamber than the middle. During S9–S10A, pulsed basal contractions in the middle of the egg chamber squeeze the tissue outward. During the last stages of oogenesis (represented by S12), basal cell surfaces maintain contact with the ECM and exert tension on the tissue. The aspect ratio (length to width) of each stage is shown at the far left.

of *ADAM metallopeptidase with thrombospondin type 1 motif A* (*AdamTS-A*); loss of function of the encoded matrix metalloprotease yields round egg chambers, likely by altering the relative stiffness of the basement membrane (Wittes and Schüpbach 2018).

A second process, egg chamber rotation, overlaps temporally with these apical contractions and also relies on information from the poles. Beginning at S1, egg chambers build a basal actin network oriented across the plane of the entire epithelium (Gutzeit 1990, 1991). By creating this unconventional form of planar polarity (see *Unconventional PCP facilitates tissue rotation and egg chamber elongation*), the follicle cells can coordinate their movement to rotate the entire egg chamber within the ovariole. Each egg chamber moves independently of its neighbor (and sometimes in opposite directions) by modulating adhesion with the interconnecting stalk cells, which are stationary and remain connected to the outside of the basement membrane of each

neighboring egg chamber (Haigo and Bilder 2011; Cetera et al. 2014). As the follicle cells crawl along the basement membrane, they secrete additional collagen, laminin A, and other ECM components to build up the mesh such that it is also oriented within the plane (Gutzeit et al. 1991; Schneider et al. 2006; Haigo and Bilder 2011; Cetera et al. 2014; reviewed by Isabella and Horne-Badovinac 2015a, 2016; Loza et al. 2017). At first (S1–S5) the rotation is slow, but the tempo increases during S6–S8, and the movement of the egg chamber within this structured ECM framework elongates the egg chamber like a baker rolling out a ball of dough. Unlike the baker's bread dough, however, the egg chamber is continually growing, and this growth fuels expansion, particularly at the poles where resistance from the ECM is low (Gates 2012; reviewed by Bilder and Haigo 2012; Cetera Horne-Badovinac 2015). Surprisingly, overall egg chamber polarity (i.e. the oocyte at the posterior) depends on the correct regulation of these follicle cell–ECM interactions (Loza et al. 2017).

A third process begins in early S9, after egg chamber rotation ceases, and continues through S10. In this case, the basal surfaces of the follicle cells exhibit coordinated actomyosin contractions, pulsatile in nature and oriented in rings around the circumference of the egg chamber (Gutzeit 1991; He et al. 2010). This behavior squeezes the growing egg chamber like a corset, restricting expansion of the diameter while allowing an increase in length. Although myosin activity is regulated cell autonomously through the Rho kinase pathway (He et al. 2010), the follicle cells employ nonautonomous interactions between integrins and the ECM to regulate Rho activity within the epithelial plane (Qin et al. 2017). Anchored and activated by this integrin–matrix connection, the follicle cells extend filopodia across neighboring cells and form supracellular basal stress fibers, thereby coordinating the production of mechanical tension across the entire egg chamber (Mateos et al. 2020; Popkova et al. 2020).

Finally, at S11, nurse cell dumping enlarges the oocyte, and the egg chamber continues to lengthen during subsequent stages (see *Nurse cell dumping and degradation*; King 1970). Loss-of-function mutations in Dystrophin and Dystroglycan, which form a complex that links the actin cytoskeleton to the ECM, disrupt basal actin organization and the ECM and lead to a reduction in egg chamber length during S12–S14 (Cerqueira Campos et al. 2020). Similarly, RNAi studies suggest that core septate junction components contribute to egg chamber elongation and also impact morphogenesis of cells synthesizing specialized eggshell structures (Alhadyian et al. 2021).

In these ways, follicle cell–matrix interactions create a "molecular corset" that restrains the growing egg chamber and forces its elongation in an anterior-posterior direction.

Unconventional PCP facilitates tissue rotation and egg chamber elongation

The transformation of the round egg chamber to an ellipse involves an ~2.5-fold change in aspect ratio that occurs mainly during S6–S9 (Haigo and Bilder 2011). To understand this change, scientists have focused on the collective cell migration that predominates during this period. Interestingly, mammary gland cell lines form cysts in 3D matrigel and rotate using a similar mechanism (Squarr et al. 2016).

During egg chamber rotation, the follicle cells must decide on a direction so that they can coordinate their movement, but unlike many other cell migrations, this tissue has no obvious leading edge (reviewed by Uechi and Kuranaga 2018). The cue for direction begins with a symmetry-breaking event in the germarium when the protocadherin Fat2 (encoded by *kugelei*) helps to orient the growth of MTs within the follicle cells. This process provides chirality to the epithelium (Viktorinová and Dahmann 2013; Chen et al. 2016).

Subsequently, at S1, Lar receptor tyrosine phosphatase (encoded by *bola*) works with integrins and Fat2 to initiate formation of the basal actin network across the epithelium (Bateman et al. 2001; Frydman and Spradling 2001; Viktorinová et al. 2009; Cha et al. 2017). Once egg chambers exit the germarium, Fat2 also coordinates the movement of cells to initiate rotation of the epithelium; this rotation occurs perpendicular to the A/P axis (Aurich and Dahmann 2016; Viktorinová et al. 2017) and in the opposite direction of the earlier plus-end-directed MT growth (Viktorinová and Dahmann 2013; Chen et al. 2016).

As migration begins, basal protrusions move cells forward along the ECM. Gain-of-function and loss-of-function studies show that while cells are moving, the basal actin stress fibers assemble new material at the leading edge and disassemble actin at the trailing edge; this treadmilling is mediated in part by the formin encoded by *disheveled associated activator of morphogenesis* (*DAAM*; Sherrard et al. 2021). In addition, clonal analyses demonstrate that Lar acts on the leading side of cells to induce retraction of membrane in the cells ahead, while the STE20-kinase Misshapen is needed on the trailing side to release follicle cell–matrix connections in the cells that follow (Lewellyn et al. 2013; Barlan et al. 2017). Signaling by Fat2 from the trailing side to the leading side recruits the WAVE complex to basal membranes, where it interacts with Lar to link the cytoskeleton to the membrane. Based on its role in other tissues, Lar likely also links the cytoskeleton, via integrins, to the ECM (Tootle et al. 2011; Cetera et al. 2014; Squarr et al. 2016; Barlan et al. 2017; Williams et al. 2022).

Fat2 and Lar also interact with other signaling complexes that are localized in a planar polarized fashion. Mosaic analyses, immunostaining, and RNAi studies suggest that the transmembrane protein Semaphorin5c (Sema5c) and its receptor PlexinA (PlexA), well known for their roles in axon guidance and other migratory processes (Jongbloets and Pasterkamp 2014), localize to leading and trailing edges of cells, respectively; there they interact with Fat2 or Lar to coordinate cell movement (Stedden et al. 2019; Williams and Horne-Badovinac 2023).

During migration, the follicle cells secrete basement membrane proteins oriented in the plane of their movement. Polarized secretion mediated by Stratum, Rab8, Rab10, and Crag (calmodulin-binding protein related to a Rab3 GDP/GTP exchange protein) ensures basal placement of molecules (Denef et al. 2008; Lerner et al. 2013; Devergne et al. 2017). Directionality depends on high levels of phosphatidylinositol 4,5-bisphosphate (PIP2) in apical and lateral membranes, which acts in part by affecting the localization of Crag (Devergne et al. 2014). Similarly, kinesin-based transport of Rab10-containing secretory vesicles along polarized MTs facilitates basal deposition of Col4a1 (Zajac and Horne-Badovinac 2022). In contrast, SPARC (Secreted protein, acidic, cysteine-rich) and other endoplasmic reticulum (ER)/Golgi-associated proteins act as chaperones that control ECM protein content by negatively regulating incorporation into the matrix (Isabella and Horne-Badovinac 2015b).

Through tight spatiotemporal control of ECM protein deposition (Fig. 7), the follicle cells produce a corset that is stiff in the middle and more relaxed at the ends (Isabella and Horne-Badovinac 2016; Crest et al. 2017). Ultimately, this gradient of basement membrane stiffness facilitates egg chamber elongation, not by controlling the plane of cell divisions but by driving expansion of tissue toward the poles and by optimizing cell packing for

circumferential growth (Haigo and Bilder 2011; Loza et al. 2017; Chen, Crest et al. 2019). The degree of egg elongation impacts oviposition rates and is monitored by the anterior polar cells, which secrete matrix metalloproteinase (MMP) 1 to modulate basement membrane stiffness (Ku et al. 2023).

Live imaging approaches have driven many key observations that form the basis for our understanding of this process (Haigo and Bilder 2011; Shah and Devergne 2022). For example, the M-TRAIL method generates clones of GFP-tagged ECM proteins, allowing visualization of basement membrane deposition. Use of this technique clarified the activity of partial loss-of-function *kugelei* (Fat2) alleles and demonstrated a causal link between egg chamber rotation and egg chamber elongation (Chen et al. 2017). Atomic force microscopy of live egg chambers showed that differential stiffness across regions of the basement membrane directs egg chamber elongation (Chlasta et al. 2017; Crest et al. 2017; Chen, Crest et al. 2019). Near total internal reflection fluorescence microscopy allowed characterization of actin stress fibers and showed that their treadmilling involves formation of distinct types of adhesions at the leading and trailing edges of cells (Sherrard et al. 2021). A simple but ingenious method for mounting egg chambers allows easy exchange of culture medium, facilitating drug studies and use of lipid dyes (Zajac et al. 2023). Complementing these tools are computational methods such as imaging surface analysis environment (ImSAnE) that facilitate quantitation by virtually unrolling the epithelium into a flat sheet for morphometric analyses (Chen et al. 2016). Thus, egg chamber elongation has become a powerful system for investigating tissue morphogenesis.

Ovulation, egg activation, and successful fertilization

Once egg chambers reach the posterior end of the ovariole and become mature S14 egg chambers, encapsulated oocytes are released into the oviduct through a process called ovulation. Traveling down through the lateral and common oviduct (Fig. 1), mature oocytes are activated by hardening the eggshell, resuming meiosis, and modification of maternal mRNA and proteins for proper fertilization and embryogenesis, a process likely influenced by the microenvironment of the reproductive tract. Recent advances in this area have uncovered multiple similarities between mammalian and *Drosophila* ovulation and egg activation. Thus, *Drosophila* is an attractive genetic model system to discover and characterize novel molecules involved in ovulation, egg activation, and fertilization.

Ovulation

Cellular process of ovulation

Oocytes of S14 egg chambers are encapsulated by a thin layer of somatic follicle cells, which are essential for eggshell formation, oocyte development, and early embryogenesis (see *Eggshell production*). During ovulation, the oocyte is released into the lateral oviduct, while the follicle cells remain at the end of the ovariole (Deady et al. 2015; Mahowald 1972). Ovulation consists of 2 steps: (1) follicle trimming, in which posterior follicle cells of the S14 egg chamber reach the lateral oviduct and are broken down and (2) follicle rupture, in which the oocyte is actively squeezed out of the follicle cell capsule and into the lateral oviduct (Fig. 8; Supplementary Movie 1; Deady et al. 2015).

Once the oocyte is released into the lateral oviduct, the residual follicle cells stay at the end of the ovariole attached to the next younger egg chamber, presumably through the stalk cells (Spradling 1993). These residual follicle cells maintain anterior and posterior orientation and gene expression as they were in the S14 egg chamber and accumulate yellow pigmentation (Deady et al. 2015). Thus, these residual follicle cells are named *Drosophila* corpus luteum, analogous to the corpus luteum in mammals. Such pigmented materials are found in ovaries throughout the class Insecta, revealing shared biological processes (Büning 1994). *Drosophila* corpus luteum stays at the end of the ovariole for up to 12 hours, and its degradation may involve the oviduct epithelial cells (Nezis et al. 2002). Thus, ovulation in *Drosophila* not only consists of a follicle rupture but also results in corpus luteum formation, as occurs in mammalian ovulation (Conti et al. 2012; Fan et al. 2012). However, the function and the degradation mechanisms of the *Drosophila* corpus luteum are completely unknown.

Follicular intrinsic factors in ovulation

Follicle trimming during ovulation is driven by the activity of MMPs, a family of proteolytic enzymes that regulate ECM homeostasis (Page-McCaw et al. 2007). The *Drosophila* genome contains 2 MMP coding genes: *Mmp1* and *Mmp2*. These enzymes have nonoverlapping roles in tissue remodeling and can both be inhibited by endogenous Tissue inhibitor of matrix metalloproteinase (Timp; Page-McCaw et al. 2003). MMP2 is specifically expressed in posterior follicle cells in S14 egg chambers (Deady et al. 2015). Functional studies show that MMP2, but not MMP1, has a central role in follicle trimming and rupture (Deady et al. 2015). MMP2 is implicated in the dissociation of larval fat body cells during pupal development through modulating basement membrane and cell–cell junctions (Jia et al. 2014). Presumably, MMP2 dissociates posterior follicle cells through a similar mechanism, but the exact targets of MMP2 during follicle trimming are still unknown.

MMP2 activity is tightly regulated to ensure only one oocyte ovulates at a time. At any given time, only a single S14 egg chamber (out of more than a dozen) exhibits prominent posterior MMP activity in vivo (Deady et al. 2015). Two signaling pathways regulate MMP2 activity during the process of ovulation: ecdysone signaling and octopaminergic signaling. The monooxygenase Shade (Shd), which converts ecdysone (E) to 20E, is not detected in S13 follicle cells but is significantly upregulated in S14 follicle cells. This upregulation of Shd is essential for MMP2 activity and ovulation (Knapp and Sun 2017). In addition, EcR is required in mature follicle cells to induce MMP2 activation and ovulation. EcR is encoded by a single gene, that, through alternative splicing, produces 3 isoforms (EcR.A, EcR.B1, and EcR.B2) with identical DNA/ligand binding domains but different N-terminal regions (Talbot et al. 1993). Expression analysis and genetic rescue experiments suggest that EcR.B2, the shortest isoform, is the receptor that mediates ecdysone signaling in mature follicle cells (Knapp and Sun 2017). Thus, mature follicle cells regulate both ecdysone production and its receptor expression to fine-tune the ecdysone signaling for MMP2 activation and ovulation. It is currently unknown how EcR.B2 mediates differential ecdysone signaling from EcR.A and EcR.B1 and what downstream targets affect MMP2 activity. Although we do not know the exact mechanism for how ecdysone signaling regulates MMP2 activation, it is clear that ecdysone does not affect MMP2 expression in posterior follicle cells (Knapp and Sun 2017) nor *Oamb* (*Octopamine receptor in mushroom body*) expression in all follicle cells; this latter process mediates follicular octopaminergic signaling for ovulation (see below).

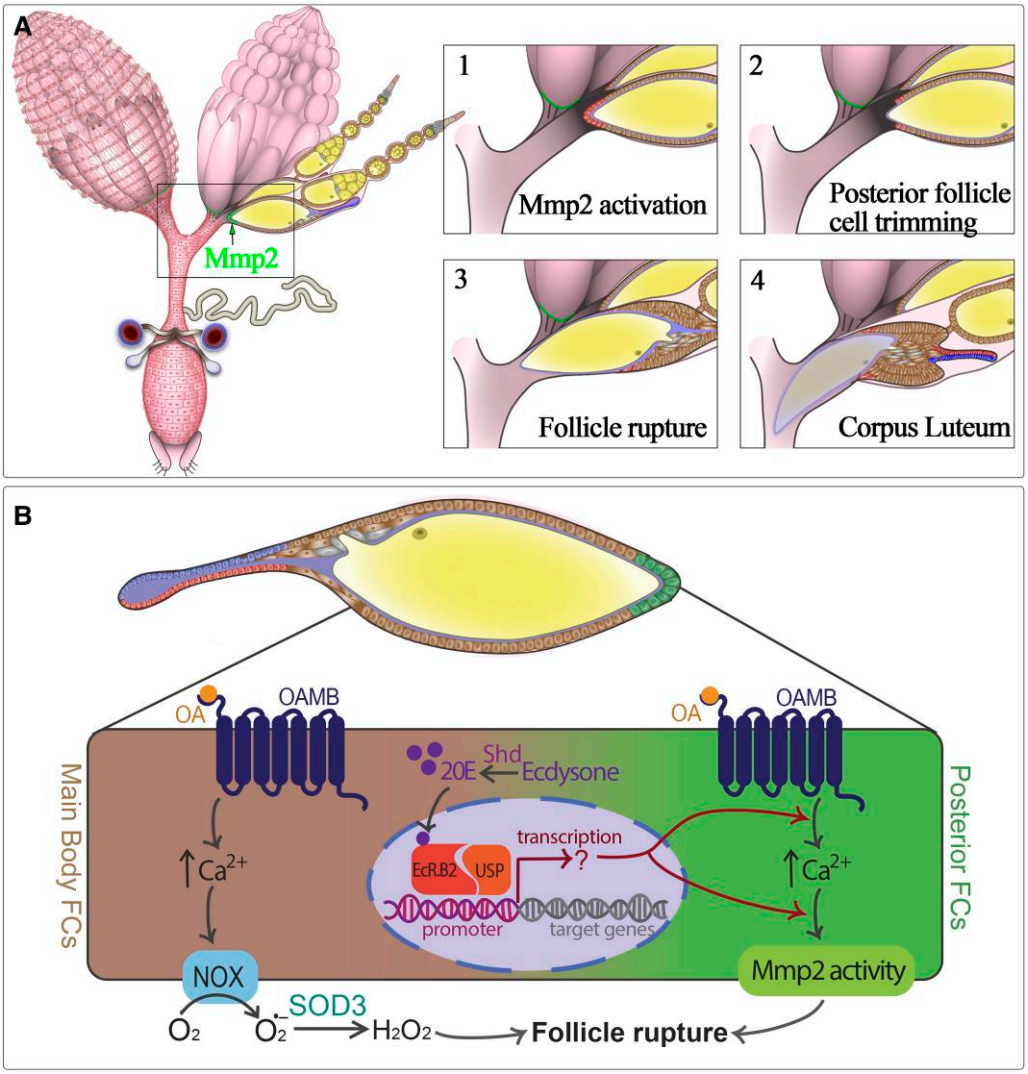

Fig. 8. Process of ovulation and intrinsic factors in ovulation. a) A model shows the key steps in ovulation (adapted from Deady et al. 2017). Ovulation begins with activation of Matrix metalloprotease 2 (MMP2) in posterior follicle cells. Activation is followed by follicle cell trimming and follicle rupture. Once the oocyte is ovulated, the follicle cell sheath is maintained as a corpus luteum whose function is completely unknown. b) Describes the intrinsic roles for ecdysone signaling, calcium signaling, reactive oxygen species, and octopamine in main body or posterior follicle cells to control ovulation.

MMP2 activity is also regulated by octopaminergic signaling. Octopamine signaling is thought to function as an equivalent to adrenergic signaling in mammals (Roeder 2005). Octopamine (OA) is produced from tyrosine by Tyrosine decarboxylase (Tdc) and Tyramine β-hydroxylase (TβH). The *Drosophila* genome contains 2 genes encoding Tdc (*Tdc1* and *Tdc2*) and one gene encoding TβH (*Tbh*). There are 4 genes (*Oamb*, *Octβ1R*, *Octβ2R*, and *Octβ3R*) encoding 5 OA receptors (*Oamb* produces 2 different splicing isoforms Oamb.K3 and Oamb.AS) (Han et al. 1998; Maqueira et al. 2005).

Several lines of evidence demonstrate that octopaminergic signaling regulates *Drosophila* ovulation. Mutation of *Tdc2* or *Tbh* leads to egg retention in the ovary and female sterility (Monastirioti et al. 1995, 1996; Cole et al. 2005). The latter can be rescued by feeding mutant flies with OA or by overexpression of TβH in a subset of abdominal ganglion neurons located at the ventral nerve cord (Monastirioti et al. 1996; Monastirioti 2003). These TβH-expressing neurons (also named octopaminergic neurons) project to the periphery where they innervate ovaries and the oviduct to regulate ovulation (Monastirioti 2003; Rodríguez-Valentin et al. 2006).

On the receiving end, Oamb is one of the OA receptors involved in ovulation, as *Oamb* mutant females are very weakly fertile, lay few eggs, and show strong egg retention in the ovary (Lee et al. 2003). Oamb is strongly expressed in oviduct epithelial cells, as well as in mature follicle cells (Lee et al. 2003, 2009), where it activates MMP2 for subsequent follicle rupture (see *Follicular extrinsic factors in ovulation* for the role of oviduct Oamb; Deady and Sun 2015). In addition, stimulation of isolated mature follicles with exogenous OA is sufficient to induce follicle trimming and rupture in an ex vivo culture system that lacks ovariole and oviduct muscle (Deady and Sun 2015; Knapp et al. 2018).

Ex vivo studies show that OA functions through the Oamb receptor in S14 follicle cells to induce the rise of intracellular Ca^{2+} and to activate MMP2 (Deady and Sun 2015). The mechanism linking the Ca^{2+} rise with MMP2 activation is still unclear, but it is not through regulating MMP2 expression (Deady and Sun 2015). One hypothesis is that the increase of intracellular Ca^{2+} leads to the secretion of MMP2, thus allowing MMP2 to degrade the ECM. MMP2 is unlikely to be the only target of the follicular adrenergic signaling because OA-induced Ca^{2+} rise spreads

across the entire follicle cell layer (Deady et al. 2015; Deady and Sun 2015).

Consistent with this idea, genetic screens identified NADPH oxidase (Nox), a member of the Nox/Duox family for superoxide production (Ritsick et al. 2007), as another target of adrenergic signaling in S14 follicle cells that is required for follicle rupture (Li et al. 2018). *Nox* mRNA is enriched in S14 follicle cells (Eichhorn et al. 2016; Li et al. 2018; Oramas et al. 2023). When OA/Oamb-induced Ca^{2+} rise activates Nox in all follicle cells of S14 egg chambers, these cells produce superoxide. Superoxide is then converted by an extracellular superoxide dismutase 3 to hydrogen peroxide, which serves as a signaling molecule for follicle rupture (Li et al. 2018). Hydrogen peroxide does not seem to affect OA-induced MMP2 activation, but it is still unclear what molecules are targeted by hydrogen peroxide during follicle rupture.

These studies and others highlight that S14 follicle cells acquire a unique molecular signature (Jevitt et al. 2020) that allows them to respond to ovulatory stimuli. Interestingly, the zinc finger transcription factor Hindsight (Hnt, AKA Pebbled) is re-upregulated in S14 follicle cells after being suppressed in main body follicle cells during S10B–S13 (Deady et al. 2015). In contrast, multiple transcription factors, including Cut, Ttk69, and Br, are downregulated in S14 follicle cells (Knapp et al. 2019). The downregulation of these transcription factors ensures proper upregulation of Hnt, which in turn upregulates Oamb in all follicle cells and MMP2 in posterior follicle cells (Deady et al. 2017).

Hnt is not required for the upregulation of Nox expression in mature follicle cells. Instead, a basic helix-loop-helix/Per-ARNT-SIM (bHLH/PAS) domain transcription factor, Single-minded (Sim), drives Nox expression, as well as Hnt, Oamb, and MMP2 expression (Oramas et al. 2023). Sim is upregulated in follicle cells transiently during S10–S12 and then again in S14. At S10, ecdysone signaling induces expression of Ttk69 and Ftz-f1; the latter one is an NR5A family nuclear receptor that directly controls Sim expression at S10–S12 to promote follicle cell differentiation (Sun et al. 2008; Knapp et al. 2020). It is unclear, however, what signals upregulate Sim expression at early S14 to promote proper transition into S14. More work will be needed to understand the final maturation of S14 egg chambers and the regulatory mechanisms of follicular intrinsic factors in ovulation.

Finally, the intrinsic molecular mechanisms controlling ovulation in *Drosophila* are highly conserved in other species, including mammals. For example, MMPs, steroid signaling, reactive oxygen species (ROS), and adrenergic signaling also play key roles in vertebrate ovulation (Curry and Smith 2006; Shkolnik et al. 2011; Richards et al. 2015; Tokmakov et al. 2020). Compounds inhibiting *Drosophila* follicle rupture can also inhibit ovulation in mouse (Jiang et al. 2021). Therefore, *Drosophila* becomes a useful model for screening nonhormonal contraceptive compounds targeting the ovulation process.

Follicular extrinsic factors in ovulation

In addition to follicular intrinsic factors, multiple extrinsic factors regulate ovulation. These factors include muscle contraction, mating, and secretions from the reproductive tract and fat body.

Muscle contractions are coordinated by 2 types of neurons. Octopaminergic neurons from the abdominal ganglion innervate the peritoneal sheath and bilateral and common oviduct muscle to form type-II neuromuscular junctions (NMJs), while glutaminergic motor neurons innervate common oviduct and uterus muscle to form type-I NMJs (Middleton et al. 2006; Rodríguez-Valentin et al. 2006; Castellanos et al. 2013). Octopamine enhances muscle contraction in the peritoneal sheath but inhibits it in the oviduct (Middleton et al. 2006; Rodríguez-Valentin et al. 2006; Rubinstein and Wolfner 2013). In contrast, glutamate acts as an excitatory neurotransmitter that stimulates oviduct muscle contraction (Rodríguez-Valentin et al. 2006). Inactivating both types of neurons leads to egg retention in the ovary and defective ovulation, while ablating only the glutaminergic neurons results in egg jams in bilateral oviducts (Castellanos et al. 2013). Consistent with this observation, mutations in *transformer* (*tra*) and *dissatisfaction* (*dsf*), which control differentiation of the glutamatergic neurons that innervate the oviduct, result in similar egg jam phenotypes (Finley et al. 1997, 1998; Castellanos et al. 2013; Evans and Cline 2013; Gou et al. 2014). These studies indicate that glutamate-stimulated contraction of oviduct muscle plays a key role in moving eggs through the oviduct. It is therefore thought that the coordinated contraction of ovariole sheath and relaxation of oviduct muscle by octopamine likely facilitates the ovulation process (Middleton et al. 2006; Rodríguez-Valentin et al. 2006; Rubinstein and Wolfner 2013). In addition, recent work showed that JH promotes the assembly of ovarian muscle ECM and ovarian muscle contraction, and disruption of JH signaling reduces ovulation (Luo et al. 2021). Despite these recent advances, many questions regarding the role of muscle contraction in ovulation still remain. In particular, it is unclear how octopamine differentially regulates peritoneal and oviduct muscle contraction.

Ovulation is greatly induced upon mating, although virgin females can ovulate at a slower rate. Mating induces a profound change in female reproductive behaviors for several days, including increased ovulation, egg production, and sperm storage, and reduced sexual receptivity and lifespan, as well as a change in feeding behavior (Kubli 2010; Kubli and Bopp 2012). In addition, mating enhances neurotransmitter release in the female reproductive tract, stimulates the maturation of oviduct epithelium that lines the lumen, and induces the relaxation of oviduct musculature; these changes are likely responsible for the increased rate of ovulation and egg laying (Heifetz and Wolfner 2004; Kapelnikov, Rivlin et al. 2008; Kapelnikov, Zelinger et al. 2008; Rubinstein and Wolfner 2013; Heifetz et al. 2014).

Products of the male's accessory gland are necessary for all or most of these postmating responses. In particular, 2 male accessory gland proteins, Acp26Aa (Ovulin) and Acp 70A (sex peptide, SP), are responsible for the induction of ovulation and egg laying (Chen et al. 1988; Aigaki et al. 1991; Herndon and Wolfner 1995; Heifetz et al. 2000; Peng et al. 2005). Upon entering the female reproductive tract, Ovulin stimulates egg laying in females for the first day by increasing the ovulation rate immediately after mating (Herndon and Wolfner 1995; Heifetz et al. 2000, 2005). Ovulin's effect on ovulation is partly through enhancing octopaminergic signaling and relaxing oviduct musculature (Rubinstein and Wolfner 2013); however, it is still unknown what receptor mediates Ovulin's effect.

In contrast to Ovulin, SP is the primary seminal peptide that mediates both short- and long-term postmating responses (Chen et al. 1988; Kubli 2003). Females mated with SP-deficient males behave like virgin females except they exhibit a very weak and transient increase in egg laying and a decrease of sexual receptivity in the first 24 h after mating (Aigaki et al. 1991; Chapman et al. 2003; Liu and Kubli 2003). The residual stimulatory activity of SP-deficient males is likely due to the activity of Ovulin and Dup99B (Ductus ejaculatorius peptide 99B), an ejaculatory ductal peptide able to bind to the SP receptor (SPR; Saudan et al. 2002; Yapici et al. 2008). With the help of other male seminal proteins (Ram and Wolfner 2009), SP binds to sperm via its N-terminal

end; there, its C-terminal end, known to be essential for postmating responses, is gradually released from stored sperm by cleavage at a trypsin cleavage site, thus prolonging the postmating responses (Peng et al. 2005).

SP induces postmating behavior changes through SPR, a G protein-coupled receptor highly expressed in the common oviduct, the SPT, and the sensory neurons innervating the reproductive tract (Yapici et al. 2008). Genetic studies identify the SP-sensing neurons as 2 bilateral clusters of 3 SPR^+ neurons coexpressing *pickpocket* (*ppk*), *dsx*, and *fruitless* (*fru*) (Yang et al. 2009; Hasemeyer et al. 2009; Rezával et al. 2012). These SP-sensing neurons reside on the anterior uterus and project to a subset of dsx^+ interneurons at the ventral nerve cord. The interneurons relay the signal to a higher order brain center for signal integration and ultimately to a subset of 9 *dsx*+ octopaminergic neurons that innervate the female reproductive system (Rezával et al. 2012; Rezával et al. 2014; Feng et al. 2014; Wang, Wang et al. 2020). Thus, mating ultimately leads to increased octopamine release (Heifetz et al. 2014), which is responsible for increased ovulation and egg laying.

Furthermore, secretions from oviduct epithelia, fat body, and reproductive-tract glands (SPT and parovaria) may also regulate ovulation. The entire oviduct is lined by a monolayer of epithelial cells that are joined by septate junctions along their lateral membranes to seal the oviduct lumen (Kapelnikov, Rivlin et al. 2008). *Oamb* and *Octβ2R* are highly expressed in oviduct epithelial cells and are required in these cells for normal ovulation (Lee et al. 2003, 2009; Lim et al. 2014; Li et al. 2015). This adrenergic signaling in the oviduct epithelium activates both cAMP-PKA and Ca^{2+}-CaMKII signaling pathways, which are then thought to induce secretion of fluids needed for ovulation (Lim et al. 2014). In addition to the oviduct, a fat body-derived neuropeptide, CNMamide, also promotes ovulation, likely by acting on the brain center that ultimately regulates octopaminergic neurons innervating the female reproductive tract (Grmai et al. 2023). Furthermore, the SPT and parovaria also play important roles in ovulation, likely through the secretion of key factors (see *Female reproductive tract secretions and reproductive success* for details).

In summary, octopamine is the key signal that induces ovulation. It is released from octopaminergic neurons innervating the ovary and the oviduct and activates its receptors in mature follicle cells, oviduct epithelium, and ovarian and oviduct muscle. Mating increases ovulation rate by enhancing octopamine release. Other environmental factors, e.g. circadian rhythm, and female physiological status can also influence ovulation rate and likely function by influencing the octopamine signaling pathway (Manjunatha et al. 2008). This hypothesis is supported by the recent discovery that bacterial infection can acutely decrease ovulation rate through a NF-κB-dependent mechanism that dampens octopamine release from octopaminergic neurons (Kurz et al. 2017). Despite recent progress, many questions still remain in the ovulation field. For example, how do octopaminergic neurons precisely activate MMP2 in only one mature follicle and how are mature follicles selected for ovulation among multiple ovarioles in the same ovary and between 2 ovaries?

Egg activation

The end result of ovulation is the release of the mature oocyte into the oviduct. The passage of the mature oocyte through the oviduct into the uterus accompanies egg activation, a process transforming the mature oocyte into the haploid egg that is able to sustain embryogenesis upon fertilization. In contrast to egg activation in mammals, where sperm entry is the trigger, egg activation in *Drosophila* is triggered by osmotic/mechanical pressure when passing through the narrow lumen of the oviduct (Horner and Wolfner 2008a). Consistent with this idea, eggs in the uterus are better activated than those in the oviduct (Heifetz et al. 2001), and laid unfertilized eggs are fully activated (Doane 1960). Mature oocytes are dehydrated at the end of oogenesis (Drummond-Barbosa and Spradling 2004) and can be artificially activated in vitro with hypotonic buffer and/or hydrostatic pressure (Mahowald et al. 1983; Page and Orr-Weaver 1997; Horner and Wolfner 2008b).

Despite the different triggers for egg activation between *Drosophila* and vertebrates, the downstream events for egg activation are highly conserved including (1) a rise of intracellular calcium (calcium wave), (2) physical and chemical changes to the oocyte's outer coverings (eggshell hardening), (3) resumption and completion of meiosis to form a haploid female pronucleus (meiotic resumption), (4) dynamic changes in maternal mRNA and proteins (maternal mRNA/protein processing), and (5) cytoskeletal rearrangement. Multiple excellent reviews have been published in the past 10 years (Pesin and Orr-Weaver 2008; Horner and Wolfner 2008a; Tadros and Lipshitz 2009; Stetina and Orr-Weaver 2011; Krauchunas and Wolfner 2013; Sartain and Wolfner 2013; Laver et al. 2015; Avilés-Pagán and Orr-Weaver 2018). Here, we briefly summarize the current understanding of egg activation.

Calcium wave

Earlier work shows that Ca^{2+} in hypotonic buffer is essential for egg activation in vitro (Horner and Wolfner 2008b). A genetically encoded Ca^{2+} sensor reveals a single, rapid calcium wave during egg activation in vitro and in vivo, a wave that starts at either the posterior pole or both poles of the oocyte and propagates across the entire oocyte cytoplasm within several minutes (Kaneuchi et al. 2015; York-Andersen et al. 2015). The Ca^{2+} ions are likely derived from perivitelline space. The rehydration of the oocyte due to osmotic pressure, as opposed to the mechanical pressure from the passage through the narrow oviduct, likely initiates the Ca^{2+} wave (York-Andersen et al. 2021). Both pharmacological and genetic experiments showed that mechanosensitive Transient receptor potential cation channel, subfamily M (TrpM) is the calcium channel for external calcium influx into oocytes during egg activation in vitro (Kaneuchi et al. 2015; Hu and Wolfner 2019; York-Andersen et al. 2021). Unexpectedly, eggs laid by *TrpM* germline KO females are still activated, albeit embryo hatch rates are reduced (Hu and Wolfner 2019). One potential explanation for this result is that additional channels are involved in calcium influx into oocytes in vivo.

The propagation of the calcium wave requires both release of internal ER calcium stores and actin cytoskeleton remodeling. Disruption of IP3R (inositol 1,4,5,-triphosphate receptor) or PLC21c (phospholipase C at 21C), 2 important components in ER calcium release, compromises the calcium wave propagation but does not affect the wave initiation (Kaneuchi et al. 2015; Hu et al. 2020). Treating oocytes with cytochalasin D, an actin polymerization inhibitor, also leads to a stuttered calcium wave, which retracts prematurely and never encompasses the whole oocyte (York-Andersen et al. 2015). It is still unclear how the TrpM-mediated external calcium leads to activation of PLC21c and actin cytoskeleton remodeling and how these events help the propagation of the calcium wave.

Eggshell hardening (egg swelling/eggshell crosslinking)

Eggshells from laid eggs are completely insoluble and water impermeable. The process of eggshell hardening is another area

with large gaps in our knowledge. The eggshell matrix becomes recalcitrant to denaturing solutions at the end of oogenesis through the activity of peroxidases that crosslink the chorion on tyrosine residues (Petri et al. 1976). Crosslinking initiates at the poles and moves toward the central part of the eggshell. Recent studies identified candidate enzymes (Konstandi et al. 2005; Fakhouri et al. 2006; Tootle et al. 2011), including Pxd (Peroxidase), whose transcripts are enriched at anterior and posterior poles, potentially explaining the wave of hardening that initiates at these sites. Peroxidases reside in the inner chorion layer and endochorion as early as S11, but they become active only after secretion of hydrogen peroxide by the follicle cells at S14 (Margaritis 1985).

Vitelline membrane proteins also remain soluble until S14, but at S10B, several events occur to initiate maturation of the vitelline envelope. Genetic, biochemical, and cell biological studies demonstrate that the microvilli that protrude from the oocyte and follicle cells and that separate the vitelline membrane-containing droplets retract, allowing the droplets to coalesce (D'Alterio et al. 2005; Schlichting et al. 2006; Romani et al. 2016); some vitelline membrane proteins are processed (Pascucci et al. 1996), and some form disulfide bridges (Wu et al. 2010). A key regulator of these changes is Palisade (encoded by *psd*), which exhibits unique structural features compared to other vitelline membrane proteins (Popodi et al. 1988; Elalayli et al. 2008).

As a result of these changes at the end of S10B, large macromolecules can no longer pass into the oocyte, preventing the use of traditional in situ hybridization methods to analyze transcripts. Clever alternatives help circumvent this impermeability, including live image analysis (Forrest and Gavis 2003) and modifications of treatments used to analyze transcripts in fixed embryos (Ali-Murthy and Kornberg 2016). Note that the oocyte can still take up molecules that had been stored in the vitelline membrane (Noguerón et al. 2000).

The timing of this first phase of vitelline membrane maturation coincides with the termination of patency, the remodeling of tricellular junctions in the follicular epithelium that allows passage of molecules from the hemolymph into the oocyte (see *Lipid accumulation and storage*). These transitions might be regulated by Ttk69, since partial loss of *ttk69* function disrupts both vitelline membrane integrity (French et al. 2003) and the cessation of patency (Row et al. 2021).

Biochemical analyses of deletion and amino acid substitution mutants demonstrate that at S13 and S14, extensive crosslinking generates covalent bonds between precisely spaced sulfhydryl groups of the major vitelline membrane proteins (Andrenacci et al. 2001; Wu et al. 2010). Similar types of structure—function studies suggest that a hydrophobic region at the amino terminus aligns the VM domains to facilitate crosslinking (Manogaran and Waring 2004). These disulfide bonds maintain the integrity of the eggshell during passage of the egg through the oviduct, where peroxidase-dependent crosslinking makes the vitelline membrane irreversibly insoluble and bleach resistant (Petri et al. 1976; Heifetz et al. 2001). Proper vitelline membrane maturation requires the activity of 3 other secreted proteins, Alpha methyl dopa-resistant (Amd), Yellow-g, and by inference, Yellow-g2; in these cases, drug inhibitor studies, mutant phenotypes, or comparisons to known functions of other family members suggest that these proteins might catalyze one or more of these late crosslinking steps (Konrad et al. 1993; Claycomb et al. 2004).

Meiosis resumption and completion

Early in oogenesis, oocytes enter meiosis and are arrested in meiotic prophase I. They resume first meiotic division with nuclear envelope breakdown (also referred to as germinal vesicle breakdown) during oocyte maturation from stages 12 to 14 (Fig. 2). As described extensively in several recent reviews (Stetina and Orr-Weaver 2011; Avilés-Pagán and Orr-Weaver 2018; Hughes et al. 2018), oocyte maturation is controlled by factors such as Polo kinase, Endosulfine, Cyclin A, Cyclin B3, and Cyclin-dependent kinase 1 (Cdk1). At the end of oocyte maturation in S14 egg chambers, oocyte centrosomes are depleted through reduction of Polo kinase and pericentriolar matrix; a specialized acentrosomal meiotic spindle is assembled with the active involvement of chromosomes and kinesin motor proteins (Pimenta-Marques et al. 2016; Radford et al. 2017). Ultimately, the oocyte arrests in metaphase I at the end of oogenesis (Fig. 2).

As part of egg activation, ovulated oocytes resume meiotic division while moving from the oviduct into the uterus, and they proceed to finish 2 meiotic divisions in the uterus (Heifetz et al. 2001). One of the key regulators that transduce the egg activation signal to resume meiosis is Sarah, the *Drosophila* homolog of regulator of Calcineurin (calcipressin). Eggs laid by *sarah* mutant females are arrested at anaphase I (Horner et al. 2006; Takeo et al. 2006). Upon egg activation, Sarah is phosphorylated at Ser215 by glycogen synthase kinase 3 (GSK3; encoded by *shaggy*; Takeo et al. 2010, 2012). It is currently unknown how GSK3 is activated upon egg activation and whether it requires the rise of intracellular Ca^{2+}.

The phosphorylation of Sarah at Ser215 leads to activation of Calcineurin, a conserved Ca^{2+}/Calmodulin-dependent serine/threonine phosphatase. Calcineurin acts as a heterodimer of catalytic A (CnA) and regulatory B (CnB) subunits (Rusnak and Mertz 2000). Although the *Drosophila* genome contains 3 genes (*CanA1*, *Pp2B-14D*, and *CanA-14F*) encoding CnA subunits and 2 genes (*CanB* and *CanB2*) encoding CnB subunits, expression studies (Takeo et al. 2006) and genetic knockouts (Takeo et al. 2010, 2012) demonstrate that only *Pp2B-14D*, *CanA-14F*, and *CanB2* are required for eggs to proceed past anaphase I. The 2 catalytic subunits act redundantly in this process. Both Sarah and Calmodulin interact with CnA subunits and form stable complexes with Calcineurin in both oocytes and activated eggs (Takeo et al. 2012). It is unknown how phosphorylation of Sarah leads to activation of Calcineurin, presumably through inducing conformational changes of the complex. The activation of Calcineurin will lead to dephosphorylation of its downstream targets, some of which may be important for meiosis progression (Zhang et al. 2019).

The successful progression into anaphase I and completion of meiosis II requires activation of anaphase promoting complex/cyclosome (APC/C), an E3-ubiquitin ligase (Pesin and Orr-Weaver 2008). APC/C ubiquitinates target proteins such as Cyclin B/B3 and Securin for proteasome degradation and leads to anaphase progression (Swan and Schüpbach 2007). APC/C activity in activated eggs depends on *cortex* (*cort*), which encodes a member of the Cdc20 family of APC/C adaptors that is female germline specific (Swan and Schüpbach 2007; Pesin and Orr-Weaver 2007). Cort plays partially redundant roles in meiosis I with another member of the Cdc20 family, Fizzy (Fzy), but nonredundant roles in meiosis II (Swan and Schüpbach 2007). The evidence suggests that Cort mediates destruction of Cyclin B in the spindle mid zone at metaphase II, while Fzy mediates destruction of Cylin B along the spindle at anaphase II (Swan and Schüpbach 2007). In addition, Cort itself is a target of APC/C for degradation at the end of meiosis and is likely recognized by Fzy (Pesin and Orr-Weaver 2007).

Activation of Cort and Fzy likely depends on the Sarah/Calcineurin-mediated phosphorylation changes. Proteomic

analysis showed significant changes of Fzy phosphorylation upon egg activation in a Calcineurin-dependent manner, similar to activation of Xenopus Cdc20 during egg activation (Mochida and Hunt 2007; Zhang et al. 2019). In addition, Cort from mature oocytes appears as a doublet on western blots but as a single, weak, low-mobility band from early embryos (Krauchunas et al. 2013). This change of Cort protein pattern depends on Sarah/Calcineurin. Further investigation will be required to establish the connection between Sarah, Calcineurin, and Cort.

Maternal mRNA/protein modification

The oocyte genome is transcriptionally quiescent during egg activation, and all cellular changes for egg activation rely on posttranscriptional and posttranslational modifications. Posttranslational modification of maternal proteins, such as phosphorylation/dephosphorylation, is not limited to meiotic regulators as mentioned above. A recent phosphoproteomic analysis estimated that hundreds of proteins change their phosphorylation state upon egg activation (Krauchunas et al. 2012; Zhang et al. 2019). Interestingly, egg activation is accompanied by more dephorsphorylation events than phosphorylation events, consistent with the major role of Sarah and Calcineurin in egg activation. Indeed, Sarah/Calcineurin signaling is required for the dephosphorylation of multiple proteins including Giant nuclei (Gnu), a regulatory protein for the serine/threonine kinase Pan Gu (Png) (see below; Krauchunas et al. 2013; Zhang et al. 2019). It is unlikely that Calcineurin is the only phosphatase responsible for dephosphorylation of maternal proteins, as dephosphorylation of MAPKs is not regulated by Sarah (Sackton et al. 2007).

Extensive changes in maternal mRNA translation occur during egg activation; the majority of these changes are controlled by the Png kinase complex, which is composed of the catalytic subunit Png and 2 activating subunits Gnu and Plutonium (Plu) (Kronja et al. 2014). The Png complex is inactive in mature oocytes due to the phosphorylation of Gnu by CycB/Cdk1. Upon egg activation, degradation of CycB leads to the dephosphorylation of Gnu in a Calcineurin-dependent manner and thus the assembly and activation of Png complex. The Png complex in turn promotes translation of CycB, thus facilitating entry into the first embryonic mitosis, and the translation of *smaug*, thereby initiating the degradation of many maternal mRNAs (Tadros et al. 2007; Vardy and Orr-Weaver 2007; Krauchunas et al. 2013; Hara et al. 2017). The Png complex also promotes the degradation of Gnu and thus restricts Png complex activity to a narrow time window used for massive translational changes via direct phosphorylation and inactivation of multiple translational repressors, such as Trailer hitch (Tral) and Pumilio (Pum) (Hara et al. 2018). The influence of CycB/Cdk1 on Png complex activation also coordinates these changes with the meiotic completion.

In addition to Png-mediated phosphorylation and inactivation of translational repressors, egg activation also leads to Ca^{2+}-dependent dispersion of the processing body (P body), which is the site of translational repression (Horner et al. 2006; Weil et al. 2008, 2012; York-Andersen et al. 2015). It is unknown whether P-body dispersion is linked to phosphorylation of translation repressors by the Png complex. Furthermore, polyadenylation of multiple maternal mRNAs occurs during egg activation in a Wispy-dependent manner; however, selective poly(A)-tail shortening is the primary cause of translation changes during egg activation (Cui et al. 2013; Lim et al. 2016; Eichhorn et al. 2016). Due to the lack of genetic tools, it is much harder to decipher the exact downstream events in terms of protein and mRNA processing after egg activation.

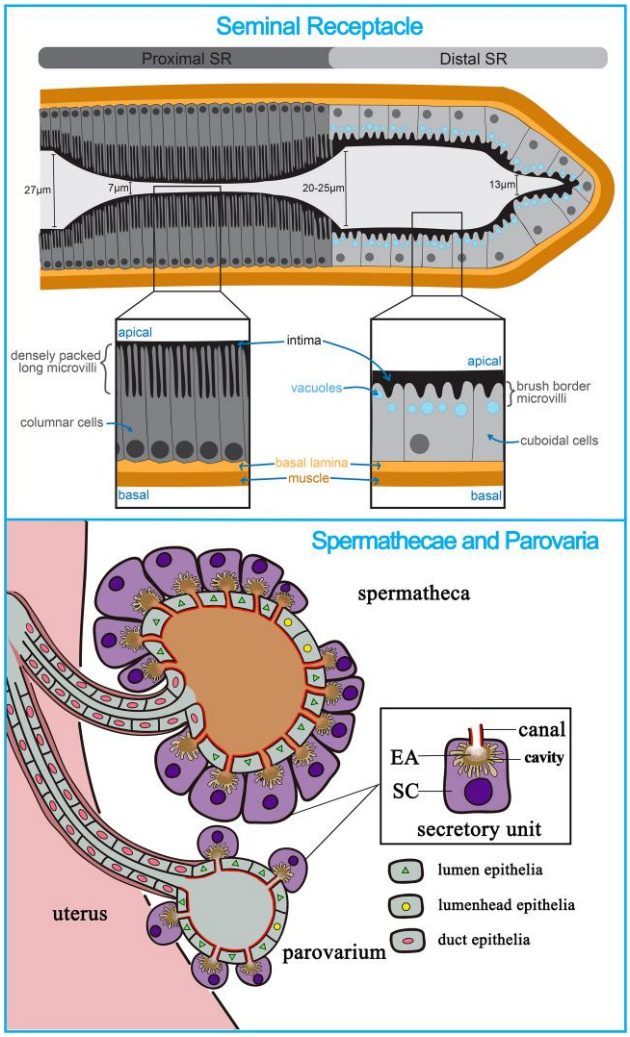

Fig. 9. Anatomical structure of seminal receptacle, spermathecae, and parovaria. The top panel shows the anatomy of the proximal and distal seminal receptacle. The bottom panel shows the anatomy of the spermathecae and parovarian structures of the reproductive tract. The secretory unit consists of a secretory cell (SC), acellular end apparatus (EA), secretory cavity (space between the apical surface of SC and EA), and a canal that connects the secretory cell to the lumen. The cuticular intima lining the lumen and canal is highlighted in red.

Female reproductive gland secretions and reproductive success

Once the egg is activated in the oviduct and lodged in the uterus, it is fertilized by a single sperm stored in 1 of 2 distinct sperm storage organs: SR and SPT (Figs. 1 and 9). The SR is considered as the primary, short-term sperm-storage organ and holds 3 quarters of stored sperm, while the SPT is considered as the long-term storage organ (Pitnick et al. 1999; Bloch Qazi et al. 2003; Manier et al. 2010).

The female reproductive tract must provide the appropriate microenvironment for proper storage and utilization of sperm to maximize fertilization success. In addition to the epithelium that lines the reproductive tract, the *Drosophila* female reproductive tract is equipped with 2 types of polyploid secretory cells surrounding the head capsules of spermathecae and parovaria (Fig. 9), and their secretions are essential for ovulation and sperm storage, thus influencing reproductive success (Allen and Spradling 2008; Schnakenberg et al. 2011; Sun and Spradling 2013). In this section, we will briefly discuss the role of glandular

secretions in sperm storage and ovulation. We direct readers to the following papers for a comprehensive review of sperm storage and the fertilization process (Schnakenberg et al. 2012; Loppin et al. 2015).

Anatomical and cellular structure of sperm storage organs and their development

The SR is a compactly coiled tube localized to the anterior ventral end of the uterus and below the common oviduct (Fig. 1). It is a dead-end tubule that branches off the oviduct wall near its junction to the uterus and measures ~2 mm in length, slightly longer than sperm, which are about 1.9 mm (Fig. 1; Pitnick et al. 1999). The SR is a heterogeneous structure with a variable lumen size across its length and distinct features in its proximal and distal halves (Fig. 9; Pattarini et al. 2006). The proximal half of the tube consists of a columnar epithelium with long and densely packed microvilli, and the distal portion consists of a cuboidal epithelium with brush border microvilli (Heifetz and Rivlin 2010). These structural characteristics suggest that the proximal and distal SR epithelia are functionally different from each other. Although expression sequence tag, microarray, RNAseq, and mass spectrometry analyses identified many SR-enriched genes (Prokupek et al. 2009, 2010; McDonough-Goldstein, Borziak et al. 2021; McDonough-Goldstein, Whittington et al. 2021), none of these genes has been assigned any function in the reproductive process. Thus, the roles of each region of the SR epithelium remain enigmatic.

Spermathecae are a pair of mushroom-shaped organs, each of which has a brown head capsule connected to the uterus by a thin epithelial duct. The 2 spermathecal ducts, surrounded by a layer of longitudinal muscle fibers, enter the dorsal uterus wall together on a low papillate elevation right behind the oviduct uterus junction (Miller 1950). The head capsule consists of a rigid lumen with a thick cuticular intima, lined by a layer of squamous epithelial cells and large cuboidal secretory cells (Fig. 9; Filosi and Perotti 1975). Slightly posterior to the spermathecae lies a pair of accessory glands named parovaria, which also have thin epithelial ducts open to the uterus wall behind spermathecal duct openings (Miller 1950). The parovarian head capsule, which is not pigmented, consists of a nonrigid lumen with a thin layer of cuticular intima surrounded by a layer of epithelial cells and fewer secretory cells.

Each secretory cell in both the spermathecae and parovaria is a separate secretory unit that discharges its secretions directly into the central lumen through its own ductule system (Fig. 9; Filosi and Perotti 1975; Allen and Spradling 2008). Each secretory unit consists of a secretory cell, an end-apparatus, and a canal (Fig. 9). The canal is a tubular invagination of the lumen cuticular intima. The terminal end of the canal is embraced by and open to the end apparatus, which consists of a network of highly branched lamellae and filamentous materials (Filosi and Perotti 1975; Mayhew and Merritt 2013). Secretory products are released into the apical space called the secretory cavity, which is defined by the microvilli of the secretory cell. They then penetrate through the end apparatus and are transported into the central lumen through the canal.

Both spermathecae and parovaria are derived from different segments of the genital disc (Keisman and Baker 2001). Early work reported that mutation of *lozenge* (*lz*), encoding a Runt domain transcription factor, causes loss of spermathecae and parovaria (Oliver and Green 1944; Anderson 1945). Lz is induced in precursors of both spermathecae and parovaria through a DsxF-dependent mechanism at the onset of pupation (Chatterjee et al. 2011; Wagamitsu et al. 2017). In addition, the zinc-finger transcription factor Glial cells missing (Gcm) acts with the NR5A family nuclear receptor Hr39 to promote precursor proliferation and bud off from the genital disc epithelium; this process forms rudimentary spermathecae and parovaria in early pupae (Allen and Spradling 2008; Sun and Spradling 2012; Cattenoz et al. 2016). These precursor cells further proliferate and utilize a defined cell lineage to build a 3-cell cluster in which asymmetric Notch signaling specifies each cell fate in this lineage (Sun and Spradling 2012; Shen and Sun 2017, 2020). The 3-cell cluster forms a concentric ring and ultimately builds the adult secretory unit (Sun and Spradling 2012; Mayhew and Merritt 2013).

The sophisticated structural arrangement of the secretory unit in spermathecae and parovaria predicts their specialized mechanisms in product secretion. Consistent with this idea, EM studies do not find traditional secretory vesicles in spermathecae and parovaria (Filosi and Perotti 1975); however, mounting evidence suggests that these cells do secrete products into the lumen. Variable amounts of electron-dense whorl-like laminae are first present in the secretory cavity and then in the spermathecal lumen (Filosi and Perotti 1975). These laminae are lipoproteins that are resistant to pronase digestion, and they are unique to spermathecae, suggesting that spermathecae and parovaria produce different secretory products and fulfill different biological functions. In addition, secretory cavity size and materials in the lumen change dynamically in females of different ages and genetic backgrounds (Filosi and Perotti 1975; Allen and Spradling 2008). Furthermore, 2 types of secretory cells are present in spermathecae: electron-dense dark cells with a well-developed rough ER and a large number of free ribosomes, and electron-clear light cells with poorly developed rough ER and cytoplasmic organelles (Filosi and Perotti 1975). Interestingly, *Hr39* transcripts are also detected in a mosaic pattern in secretory cells of spermathecae and parovaria (Allen and Spradling 2008). It is unclear whether *Hr39*-positive secretory cells correspond to dark cells or light cells as described in Filosi and Perotti (1975). Finally, a recent study suggests that a heterogenous population of extracellular vesicles is utilized by spermathecal secretory cells to release their contents to the lumen (Sanchez-Lopez et al. 2022). Despite these advances, we still do not know much about the signaling pathways controlling gland secretions.

Secretions of spermathecae and parovaria regulate fertilization success

While studying *lz* mutant females, Anderson (1945) found that spermathecae and parovaria play important roles in fertilization success, as the loss of spermathecae and parovaria in *lz* mutant female correlated with the loss of female fertility. This key role is also manifested in *Hr39* or *gcm* mutant females (Allen and Spradling 2008; Cattenoz et al. 2016). Recent studies further demonstrate that secretions from spermathecae and parovaria are not only required for efficient ovulation but also for proper sperm storage, both of which are essential for successful fertilization (Schnakenberg et al. 2011; Sun and Spradling 2013; Sanchez-Lopez et al. 2022).

Mutation of *lz* or *Hr39* disrupts the formation of spermathecae and parovaria and reduces egg laying and ovulation rate, suggesting a role of spermathecae and parovaria in ovulation (Anderson 1945; Allen and Spradling 2008; Sun and Spradling 2013). Perturbing the developmental signals for secretory cell formation demonstrates that the number of secretory cells in spermathecae and parovaria is positively correlated with the ovulation rate (Sun and Spradling 2013). In addition, Hr39 functions in adult secretory cells to regulate ovulation (Sun and Spradling 2013). These studies suggest the hypothesis that Hr39-regulated secretions from

spermathecae and parovaria influence the ovulation rate. More work will be required to identify the secreted products involved in ovulation and to decipher their precise mechanisms.

In addition to an ovulation defect, females lacking spermathecal secretory cells cannot store sperm in the spermathecal lumen, nor can they maintain sperm motility in the SR (Schnakenberg et al. 2011; Sun and Spradling 2013). These observations suggest that secretions from spermathecae function locally to attract sperm to the spermathecae and likely have a long-term effect to maintain sperm function in the SR. In addition, spermathecal secretory cells are also required for efficient sperm release from the SR, and this process depends on SPR in spermathecal secretory cells (Avila, Mattei et al. 2015). Therefore, secretions of spermathecae and parovaria have a plethora of roles in regulating sperm attraction, maintenance, and release.

The process of sperm storage is well studied in *Drosophila*, and multiple male factors have been identified (reviewed by Bloch Qazi et al. 2003; Avila et al. 2011; Schnakenberg et al. 2012). Upon copulation, ~4000 sperm are transferred to the female reproductive tract along with seminal fluid. Upon entering the uterus, sperm exhibit activated flagellar beating, move in circular foci via arc line waves, and enter the SR in a parallel, tail-leading orientation (Yang and Lu 2011; Köttgen et al. 2011). Disruption of proper flagellar beating, such as in mutant sperm of *Polycystic kidney disease 2* (*Pkd2*; Gao et al. 2003; Watnick et al. 2003), *lost boys* (*lobo*; Yang et al. 2011), and *sheepish* (*shps*; Tomaru et al. 2018), leads to fewer sperm stored in female sperm storage organs. Multiple male seminal proteins facilitate sperm entry into the sperm storage organs including Acp36DE (Accessory gland protein 36DE), which is known to regulate uterus contraction to push sperm to the sperm storage site (Neubaum and Wolfner 1999; Avila and Wolfner 2009; Avila et al. 2011). In addition, coagulation of seminal proteins at the posterior end of the semen mass forms an auto-fluorescent mating plug, which also facilitates sperm storage and whose removal is regulated by female neuron endocrine hormone Dh44 (Diuretic hormone 44) (Avila, Wong et al. 2015; Avila, Cohen et al. 2015; Lee et al. 2015; Arthur et al. 1998). Ultimately, ~25% of transferred sperm will be stored in either SR or spermathecae and will be used to fertilize ovulated eggs. These sperm are constantly beating and can be stored for up to 2 weeks (Manier et al. 2010). A few factors regulate sperm maintenance and release, such as Wasted (Wst; Ohsako and Yamamoto 2011), Accessory gland protein 29AB (Acp29AB; Wong et al. 2008), SP (Avila et al. 2010), and tyramine/octopamine (Avila et al. 2012).

Despite these studies, many questions still remain. How do secretions of spermathecae and parovaria attract sperm? Do they function as chemo attractants to activate sperm calcium channel Pkd2 and flagella beating? Do they regulate sperm storage through activating/deactivating seminal fluid proteins? Do they provide an energy source for maintaining sperm activity in the storage organs? On the other hand, the identity of the secreted products from spermathecae and parovaria is still unknown; however, it is clear that these products are secreted through the canonical protein secretory pathway, as disruption of this pathway in the secretory cells produces the same phenotype as secretory cell-ablated females (Sun and Spradling 2013). Several genomic analyses suggest that spermathecae are abundant in transcripts encoding serine proteinases, antimicrobial peptides, antioxidants, and serpins (Arbeitman et al. 2004; Allen and Spradling 2008; Prokupek et al. 2009). Future work will be required for identifying these secreted factors and clarifying the underlying mechanism for sperm attraction and function.

Conclusions and future perspectives

In the last several decades, we have made significant progress in understanding the mechanisms of building a fertilizable egg for successful reproduction in *Drosophila*. Many conserved signaling pathways have been uncovered that regulate nutrient storage, oocyte maturation, pattern formation, cell cycle transitions, follicle cell differentiation, eggshell synthesis, egg shape, ovulation, egg activation, and fertilization. *Drosophila* oogenesis is and will continue to be an excellent model system not only for the study of reproductive biology but also for many cell biological questions in general. The breadth of knowledge of *Drosophila* oogenesis, the powerful and sophisticated genetic tools available, the ease of live imaging, and the short generation time provide *Drosophila* researchers with many advantages to tackle the multiple remaining questions we highlighted at the end of each section. A comprehensive knowledge of *Drosophila* oogenesis will undoubtedly advance our understanding of many biological processes and improve human health in the near future. We hope this chapter serves as a summary of previous findings and a foundation to launch new discoveries in this field.

Data availability

The data underlying this article are available in the article and in its online supplementary material.

Supplemental material available at GENETICS online.

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Conflicts of interest statement

The author(s) declare no conflict of interest.

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