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Nuclear receptors linking physiology and germline stem cells in *Drosophila*

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Abstract

Maternal nutrition and physiology are intimately associated with reproductive success in diverse organisms. Despite decades of study, the molecular mechanisms linking maternal diet to the production and quality of oocytes remain poorly defined. Nuclear receptors (NRs) link nutritional signals to cellular responses and are essential for oocyte development. The fruit fly, *Drosophila melanogaster*, is an excellent genetically tractable model to study the relationship between NR signaling and oocyte production. In this review, we explore how NRs in *Drosophila* regulate the earliest stages of oocyte development. Long-recognized as an essential mediator of developmental transitions, we focus on the intrinsic roles of the Ecdysone Receptor and its ligand, ecdysone, in oogenesis. We also review recent studies suggesting broader roles for NRs as regulators of maternal physiology and their impact specifically on oocyte production. We propose that NRs form the molecular basis of a broad physiological surveillance network linking maternal diet with oocyte production. Given the functional conservation between *Drosophila* and humans, continued experimental investigation into the molecular mechanisms by which NRs promote oogenesis will likely aid our understanding of human fertility.

1. *Drosophila* as a model for maternal physiological control of oogenesis

Obesity, metabolic disorders, and diabetes disrupt the body's natural steroid hormone milieu, negatively impacting human health. An important, but often overlooked co-morbidity of metabolic disorders in women is poor reproductive outcome (Gu et al., 2015; Roa & Tena-Sempere, 2014). The quality of preconception maternal diets is tightly correlated with reproductive success (Gaskins & Chavarro, 2018; Hohos & Skaznik-Wikiel, 2017; Luzzo et al., 2012). Maternal obesity is associated with poor oocyte quality, which manifests as increased time to pregnancy, early pregnancy loss, and congenital abnormalities in offspring (Purcell & Moley, 2011; Talmor & Dunphy, 2015). Despite the prevalence of metabolic disorders in the United States, and the clear public health relevance (Panth, Gavarkovs, Tamez, & Mattei, 2018), the molecular mechanisms connecting oocyte quality and production to nutritionally regulated endocrine signals are poorly understood.

Among the many cell-cell communication pathways used by ovarian cells to promote oocyte development, steroid hormones are essential for maintenance of fertility, including follicle assembly, oocyte maturation, and oocyte survival (Grive & Freiman, 2015). Hormones

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circulating through the blood or hemolymph of an organism create local and long-distance signaling networks by binding to nuclear receptors in target cells, affecting transcriptional change (Weikum, Liu, & Ortlund, 2018). Steroid synthesis is a complex molecular process requiring coordinated activity of multiple substrates and enzymes in gonads and other endocrine tissues (Dallel et al., 2018). Tight spatial and temporal control of steroid synthesis and steroid reception by cognate nuclear receptors must be achieved to facilitate successful oocyte production and fertilization. As such, many female reproductive disorders (such as polycystic ovarian syndrome, endometriosis, and early pregnancy loss) are associated with aberrations in steroid hormone levels or exposure to endocrine disrupting chemicals (Pivonello et al., 2020; Zhang, Wesevich, Chen, Zhang, & Kallen, 2020).

Given the complexity of hormone signaling pathways in mammals, the fruit fly, *Drosophila melanogaster*, has emerged as an excellent genetically tractable model to study the relationship between maternal nutrition and oocyte production. Female fruit flies contain two ovaries made of about 15 ovarioles, each consisting of oocytes in increasing stages of development arranged linearly from anterior to posterior (Fig. 1A and D) (Hinnant, Merkle, & Ables, 2020). The morphology of the ovary enables young female flies to lay 80–90 eggs per day (Drummond-Barbosa & Spradling, 2001). The progressive arrangement of oogenesis also allows researchers to visualize the complete development of oocytes, from their establishment as daughters of a germline stem cell population through ovulation. Female fruit flies are exceptionally sensitive to diet conditions and can be reared on specialized media to manipulate the levels of available nutrients. Moreover, *Drosophila* oogenesis can be monitored on a per-cell basis in vivo using confocal microscopy (Fig. 1A and B) and germ cells can be easily manipulated via a widely available sophisticated genetic toolkit. The ease with which *Drosophila* are reared and their remarkable similarity to human physiology and genetics make fruit flies an incredibly powerful model organism for research.

2. Nuclear receptors as molecular surveyors of physiology

Nuclear receptors (NRs) directly link dietary and hormonal signals to transcriptional responses in target cells and serve as key regulators of development, metabolism, and reproduction (Ables & Drummond-Barbosa, 2017; Bodofsky, Koitz, & Wightman, 2017; Crowder, Seacrist, & Blind, 2017; Dallel et al., 2018; Evans & Mangelsdorf, 2014; King-Jones & Thummel, 2005; Mirth, Nogueira Alves, & Piper, 2019; Pardee, Necakov, & Krause, 2011; Weikum et al., 2018). The nuclear receptor superfamily is one of the largest families of transcription factors, divided into seven subfamilies (NR0-NR6) based on domain conservation across species, including steroid receptors, thyroid hormone receptors, retinoid acid receptors, and retinoid X receptors (Bodofsky et al., 2017; Evans & Mangelsdorf, 2014; King-Jones & Thummel, 2005; Pardee et al., 2011). NRs primarily function as ligand-gated transcription factors. A conserved zinc-finger DNA-binding domain allows NRs to bind specific sequences in the promoter regions of target genes and activate or repress transcription. Small lipophilic ligands, processed from dietary lipids such as cholesterol and fatty acids, bind to a more sequence-diverse C-terminal ligand binding and dimerization domain in the receptor, promoting conformational change when ligand is bound (Bodofsky et al., 2017; Weikum et al., 2018). The ability to directly link transcriptional change with ligands whose concentrations vary with physiology allows

organisms to adapt to a wide variety of environmental conditions and stressors. NRs frequently partner with other NRs in heterodimer configurations, and their activity can be modified by the binding of additional co-factors to increase repressor or activator function. Not all NRs, however, have known natural ligands, and at least some can affect transcription in the absence of ligand. Though mammals exhibit exceptional diversity in NRs (encoded by 48 genes in humans), the *Drosophila* genome encodes 18 NR family members, representing each of the known mammalian subfamilies. Unlike humans, the *Drosophila* genome is largely free from duplications, making functional assignments to particular genes easier than in most vertebrate model organisms. Early experimental studies in *Drosophila* took advantage of the distinct hormonally controlled developmental transitions in the insect life cycle, resulting in a wealth of molecular information about steroid hormones and their physiologically relevant NRs and transcriptional responses.

3. Ecdysone signaling as a model for cell-cell communication via nuclear receptors

Perhaps the best characterized hormones in insects are the ecdysteroids, a group of polyhydroxylated steroids essential for development, growth, behavior, and reproduction. *Drosophila* synthesize the predominant hormone, ecdysone, from diet derived cholesterol and other plant sterols (Fig. 1C) (Gilbert, 2004; Gilbert & Warren, 2005; Igarashi, Ogihara, Iga, & Kataoka, 2018; Miller, 2008; Truman, 2019). Loss of the ability to convert cholesterol, such as in flies harboring genetic mutations in the *without children* (*woc*) locus, is lethal but can be rescued with the addition of the cholesterol derivative 7-dehydrocholesterol (Wismar et al., 2000; Warren, Wismar, Subrahmanyam, & Gilbert, 2001). In larvae, ecdysone is synthesized when the neuropeptide prothoracicotropic hormone signals to a specialized organ in the brain, known as the prothoracic gland (McBrayer et al., 2007; Rewitz, Yamanaka, Gilbert, & O'Connor, 2009). Ecdysone is secreted into the hemolymph, where it is converted into its active form, 20-hydroxyecdysone (20E), which is functionally and structurally analogous to mammalian hormones estrogen, progesterone, and thyroid hormone (Fig. 1C) (Gilbert, Rybczynski, & Warren, 2002; Hoffmeister, Grützmacher, & Dünnebeil, 1967; Mangelsdorf et al., 1995; Petryk et al., 2003; Uryu, Ameku, & Niwa, 2015). Although the prothoracic glands degenerate after metamorphosis, ecdysone is prevalent in adults, albeit at lower titer than in larval stages (Dai, Henrich, & Gilbert, 1991; Richards, 1981; Schwartz, Kelly, Imberski, & Rubenstein, 1985). In adults, ecdysone can be detected in all three body segments and in specific tissues, including the gut, malphigian tubules, testes, and ovaries (Bownes, 1982; Handler, 1982; Schwartz et al., 1985).

3.1 Ecdysone promotes developmental transitions and adult reproductive physiology

Ecdysone was initially identified as a regulator of molting and metamorphosis in arthropods. Pulses of ecdysone precede developmental transitions between life stages and promote critical physiological changes, including germ band retraction and head involution during the first larval instar, larval cuticle development, and programmed cell death and cell remodeling in imaginal discs during metamorphosis (Andres, Fletcher, Karim, & Thummel, 1993; Apple & Fristrom, 1991; Braquart, Bouhin, Quennedey, & Delachambre, 1996;

Charles, 2010; Jiang, Baehrecke, & Thummel, 1997; Karlson, 2003; Kozlova & Thummel, 2003; Li & Bender, 2000; Robertson, 1936; Warren et al., 2006). Embryos lacking ecdysone biosynthetic enzymes proceed through embryogenesis due to the availability of maternally deposited transcripts, but fail to make a larval cuticle, yielding a unique ghost-like phenotype (Chavez et al., 2000; Niwa & Niwa, 2014; Uryu et al., 2015). Identification of mutants in large-scale screens sharing this phenotype provided reagents necessary to identify ecdysone biosynthetic enzymes, which were collectively referred to as the "Halloween Genes" (Gilbert, 2004; Niwa & Niwa, 2014).

While the ovary appears to be a major source of ecdysone synthesis in adult females, ecdysone levels in female *Drosophila* are highly dependent on physiological status. Genetic mutant strains that lack ovaries have reduced ecdysone titer in their hemolymph, suggesting that the ovary contributes to the overall ecdysone levels in females (Bainbridge & Bownes, 1988; Tu, Yin, & Tatar, 2002). Ecdysone biosynthesis enzymes, including those encoded by *defective in the avoidance of repellents* (*dare*) and the Halloween genes, are highly expressed in ovarian follicles, suggesting that the ovary is a source of ecdysone in adult *Drosophila* (Freeman, Dobritsa, Gaines, Segraves, & Carlson, 1999; Niwa et al., 2004; Ono et al., 2006; Warren et al., 2004). Ecdysone titer increases in females post-mating in response to the male Sex Peptide protein, transferred via seminal fluid during mating, resulting in higher levels of ecdysone in female than in males (Ameku & Niwa, 2016; Ameku, Yoshinari, Fukuda, & Niwa, 2017; Avila, Sirot, LaFlamme, Rubinstein, & Wolfner, 2011; Carmel, Tram, & Heifetz, 2016; Feng, Palfreyman, Hasemeyer, Talsma, & Dickson, 2014; Hasemeyer, Yapici, Heberlein, & Dickson, 2009; Sieber & Spradling, 2015). Additionally, because ecdysone is synthesized from dietary cholesterol, the nutritional status of the fly greatly impacts ecdysone titer. Ecdysone titer increases 24–48h after eclosion in well-fed flies, and adult females starved after eclosion fail to accumulate significant levels of ecdysone (Handler, 1982; Terashima, Takaki, Sakurai, & Bownes, 2005). Interestingly, phenotypes seen in ecdysone biosynthesis mutants do not always mimic the phenotypes seen in nutrient deprived flies, suggesting that loss of adequate nutrition does not completely abolish ecdysone titer, but instead significantly lowers it (Carvalho-Santos & Ribeiro, 2018; Terashima et al., 2005).

3.2 Ecdysone exerts cellular effects via conserved nuclear receptors EcR and Usp

Ecdysone functions as a transcriptional regulator by binding to a heterodimeric receptor complex (Fig. 1C) (Billas & Moras, 2005; Thomas, Stunnenberg, & Stewart, 1993; Yao et al., 1993). In *Drosophila*, the receptor complex is encoded by two nuclear receptors: *Ecdysone Receptor* (*EcR*), an ortholog of mammalian farnesoid X and liver X receptors; and *ultraspiracle* (*usp*), an ortholog of mammalian retinoid X receptors (Henrich et al., 1994; King-Jones & Thummel, 2005; Koelle et al., 1991; Oro, McKeown, & Evans, 1990; Yao et al., 1993). Without Usp, EcR can neither bind ecdysone nor its target DNA sequences (Hill, Billas, Bonneton, Graham, & Lawrence, 2013; Yao et al., 1993). Upon forming a heterodimer, the EcR/Usp complex (henceforth referred to as EcR) regulates transcription of target genes via ecdysone response elements (EcREs). In the absence of ecdysone, EcR represses transcription and is bound by co-repressors, including Alien (a COP9 signalosome subunit) and SMRT-related and Ecdysone Receptor Interacting Protein (SMRTER) (Dressel

et al., 1999; Heck et al., 2012; Huang, Lu, Wu, Chien, & Pi, 2014; Schubiger & Truman, 2000). In the presence of ecdysone, cells actively transport ecdysone into the cytoplasm via a membrane-resident transporter belonging to the solute carrier organic anion superfamily (Okamoto et al., 2018). Binding of ecdysone to EcR promotes a conformational change in EcR in which the ligand binding region is internalized into the three-dimensional structure of the protein (Billas et al., 2003; Hu, Cherbas, & Cherbas, 2003; Schubiger, Carré, Antoniewski, & Truman, 2005). Exposure of a new protein surface allows for release of co-repressor proteins and recruitment of transcriptional co-activators, such as the highly conserved Taiman (Tai) and Absent, small, or homeotic discs 2 (Ash2) (Bai, Uehara, & Montell, 2000; Carbonell, Mazo, Serras, & Corominas, 2013; Zhang et al., 2015). Coordinated activity of EcR with nucleosome remodeling complexes, including Putzig, ISWI/NURF, and Kismet, further constrains transcriptional activity at target genes by regulating chromatin accessibility (Badenhorst et al., 2005; Kreher et al., 2017; Kugler, Gehring, Wallkamm, Kruger, & Nagel, 2011; Latcheva, Viveiros, & Marenda, 2019; Uyehara et al., 2017).

Signaling via EcR is required to regulate gene expression in a wide variety of tissues in a spatiotemporally specific manner (Beckstead, Lam, & Thummel, 2005; Gauhar et al., 2009; Gonsalves, Neal, Kehoe, & Westwood, 2011; Li & White, 2003; Shlyueva et al., 2014; Stoiber, Celniker, Cherbas, Brown, & Cherbas, 2016; Uyehara & McKay, 2019). The range of EcR-dependent cellular activities is due, at least in part, to the expression of multiple protein isoforms. The *EcR* locus contains the prototypical genetic structure of the NR superfamily, including conserved ligand-binding and zinc-finger-like DNA-binding domains (Koelle et al., 1991). Although there are seven putative transcripts of *EcR*, there are only three functional protein isoforms, denoted as EcR-A, EcR-B1, and EcR-B2 (Talbot, Swyryd, & Hogness, 1993). Alternative promoter usage results in unique spatiotemporal expression of the three isoforms. Despite the conservation in DNA-binding domains, the EcR isoforms do not appear to be functionally redundant. Each isoform can bind a specific nucleotide sequence, and carefully controlled rescue and mis-expression experiments demonstrated tissue-specificity among the three protein isoforms (Cherbas, Hu, Zhimulev, Belyaeva, & Cherbas, 2003; Davis, Carney, Robertson, & Bender, 2005; Schauer, Callender, Henrich, & Spindler-Barth, 2011; Schubiger, Tomita, Sung, Robinow, & Truman, 2003). In vivo, EcR-A appears to function predominantly as a strong repressor, while both B class isoforms are strong activators (Braun, Azoitei, & Spindler-Barth, 2009; Dobens, Rudolph, & Berger, 1991; Hu et al., 2003). Unique N-termini of the isoforms also permit differential binding by co-activators and co-repressors to the EcR/Usp complex.

Spatiotemporal specificity of the ecdysone response is also mediated through the transcriptional targets of EcR and Usp. Many of these loci were initially identified more than 45 years ago based on the unique transcriptionally regulated “puffing” of larval salivary polytene chromosomes in response to ecdysone (Ashburner, Chihara, Meltzer, & Richards, 1974; Hill et al., 2013). Initial experiments, followed by more recent whole-genome attempts to catalog the transcriptional response to ecdysone, support a hierarchical model of ecdysone signaling, wherein EcR activation promotes the rapid expression of a small number of targets (Ashburner et al., 1974; Beckstead et al., 2005; Gauhar et al., 2009; Gonsalves et al., 2011; Hill et al., 2013; Li & White, 2003; Shlyueva et al., 2014; Stoiber et

al., 2016; Uyehara & McKay, 2019). These so-called early-response genes, including a core group of genes such as *Ecdysone-induced protein 74EF* (*E74*), *Ecdysone-induced protein 75B* (*E75*), and *broad* (*br*), encode transcription factors. These activate a tissue-specific response to ecdysone by regulating a second set of targets (late-response genes). Although EcR and Usp mediate the majority of ecdysone-dependent transcriptional responses, at least six other NR genes are themselves ecdysone-responsive: *Hormone receptor 3* (*Hr3*, also called *Hr46*), *Hormone receptor 4* (*Hr4*), *Hormone receptor-like in 39* (*Hr39*), *E75*, *Ecdysone-induced protein 78C* (*E78*), and *ftz transcription factor 1* (*ftz-f1*) (King-Jones & Thummel, 2005). Intriguingly, while expression of most of these genes is induced by ecdysone, expression of *ftz-f1* is repressed by ecdysone, likely in a feedback loop with ecdysone biosynthesis genes (Parvy et al., 2005; Woodard, Baehrecke, & Thummel, 1994; Yamada et al., 2000). Moreover, some NRs exhibit their own unique ligand activation and can promote cross-regulation between receptors. For example, Ftz-f1 can promote transcriptional activation in the absence of a ligand, but requires Hr3 for full activation (Lam, Jiang, & Thummel, 1997; Lu, Anderson, Zhang, Feng, & Pick, 2013; Musille, Pathak, Lauer, Griffin, & Ortlund, 2013; Yoo et al., 2011). Similarly, while E75 is a classic early response factor, it can also be regulated by small molecules such as nitric oxide (Cáceres et al., 2011; Marvin et al., 2009; Reinking et al., 2005). NRs are dynamically expressed in *Drosophila* tissues and are regulated by a complex web of interactions, placing them in a unique mechanistic position to fine-tune reproduction with metabolic and environmental cues (Fig. 1D) (Palanker et al., 2006; Wilk, Hu, & Krause, 2013).

4. *Drosophila* oogenesis is fueled by stem cells

The development of a viable oocyte requires a coordinated network of somatic and germ cells functioning in unison to support oocyte differentiation, meiotic maturation, and the deposition of maternal mRNA, proteins, and nutrients necessary to sustain embryogenesis post-fertilization. In *Drosophila*, oogenesis begins in the most anterior region of the ovariole, called the germarium (McLaughlin & Bratu, 2015) (Fig. 1B and D). Here, two stem cell populations divide continuously in adult flies to form the cellular precursors of the germline and somatic cells that will ultimately comprise the oocyte. Germline stem cells (GSCs) are housed at the anterior of the germarium and divide asymmetrically to self-renew and produce a differentiating daughter cell called a cystoblast (Hinnant et al., 2020). Following four rounds of mitotic division with incomplete cytokinesis, the cystoblast ultimately differentiates into a 16-cell cyst, wherein one cell becomes the oocyte and the other 15 cells adopt a nurse cell fate. Follicle stem cells (FSCs) reside in the middle of the germarium and produce pre-follicle cells that encapsulate the developing germ cells (Rust & Nystul, 2020). Pre-follicle cells further differentiate into specialized follicle cell populations outside of the germarium, ultimately forming the eggshell, chorion, and associated structures. The differentiated cell daughters of both stem cell populations are essential to form an oocyte that is ready for fertilization, as loss or malfunction of GSCs or FSCs leads to loss of fertility (Lin & Spradling, 1993; Margolis & Spradling, 1995).

While nascent egg chambers are formed in the germarium, oocyte development continues through 14 distinct stages in the vitellarium (Fig. 1A and D) (McLaughlin & Bratu, 2015). Egg chambers bud from the germarium connected by specialized follicle cells called stalk

cells. In stages 2–7, egg chambers undergo rapid growth, in part due to developmentally programmed nurse cell endocycling which increases nurse cell DNA content. Follicle cells complete four to five rounds of mitotic divisions to cover the growing germline cyst in an epithelial monolayer, differentiate into a variety of specialized cell types, and switch to endocycling at stage 5 (Duhart, Parsons, & Raftery, 2017). Movement of the follicle cells and subtle rotation of the egg chamber promote elongation (Cetera & Horne-Badovinac, 2015). Stage 8 follicles undergo vitellogenesis, as yolk proteins produced by the fat body and ovarian tissues are loaded into the oocyte (Brennan, Weiner, Goralski, & Mahowald, 1982). Beginning at stage 9, a group of 6–10 follicle cells, called border cells, delaminate from the follicle cell epithelium and migrate between the nurse cells to form the oocyte anterior margin (Peercy & Starz-Gaiano, 2020). At stage 11, concomitant with continued oocyte growth, nurse cells undergo apoptosis and dump their cytoplasmic contents into the oocyte (Quinlan, 2016). During the final stages of egg chamber development, the follicle cells secrete chorion and other proteins necessary to form different layers of the eggshell, helping to protect the oocyte once it is deposited (Osterfield, Berg, & Shvartsman, 2017). At stage 14, the egg chamber is fully developed and ready to be fertilized and deposited (Mahowald & Kambysellis, 1980).

Ovarian stem cells and their differentiating daughters rely on paracrine and endocrine signaling for their development (Ables, Laws, & Drummond-Barbosa, 2012; Armstrong, 2020; Drummond-Barbosa, 2019; Laws & Drummond-Barbosa, 2017). This is perhaps best illustrated by the complex signaling network that guides GSC self-renewal and cystoblast differentiation. GSCs reside in a stem cell niche, composed of adjacent cap cells and escort cells, that provides short range signals required to balance GSC self-renewal vs differentiation (Figs. 1B and 2) (Drummond-Barbosa, 2019; Kahney, Snedeker, & Chen, 2019). GSCs are physically anchored to the cap cells and secrete Bone Morphogenetic Protein (BMP) ligands that regulate GSC self-renewal (Fig. 2) (Song, Zhu, Doan, & Xie, 2002; Wilcockson & Ashe, 2019; Xie & Spradling, 1998). To maintain the undifferentiated fate, GSCs express RNA-binding proteins that promote self-renewal by repressing transcription of the differentiation factor Bag of Marbles (Bam) (D. Chen & McKearin, 2003; Forbes & Lehmann, 1998; Jin et al., 2008; Song et al., 2004; Szakmary, Cox, Wang, & Lin, 2005; Wang & Lin, 2004). Germ cell differentiation and cyst division are thus initiated by the derepression of *bam* transcription. Bam appears to promote differentiation by limiting the production of self-renewal and adhesion factors, and stabilizing CycA expression (Chen, Wang, et al., 2009; Ji et al., 2017; Li, Minor, Park, McKearin, & Maines, 2009; Liu et al., 2017; Perinthottathil & Kim, 2011; Shen, Weng, Yu, & Xie, 2009; Tiwari, Zeitler, Meister, & Wodarz, 2019). Somatic escort cells surrounding the dividing cysts form a second signaling center that promotes germ cell differentiation, posterior movement of the cysts, and follicle cell encapsulation (Banisch, Maimon, Dadosh, & Gilboa, 2017; Decotto & Spradling, 2005; Kirilly, Wang, & Xie, 2011; Morris & Spradling, 2011). Escort cells line the outside of the anterior germarium (regions 1–2) and send long, thin cellular protrusions into the center of the germarium, around the dividing germ cells (Fig. 1B and D) (Banisch et al., 2017; Kirilly et al., 2011; Morris & Spradling, 2011). Escort cells secrete a variety of signaling molecules that promote germ cell differentiation. These signals act to sustain the

cytoskeletal structure and dynamics of escort cell protrusions and limit the range of BMP signals emanating from cap cells (Antel & Inaba, 2020; Gao et al., 2019).

5. Multifaceted roles of NR signaling in oogenesis

Like its mammalian nuclear receptor counterparts, signaling through EcR provides a crucial link between maternal diet and oogenesis (Ables et al., 2012; Chen, Breen, & Pepling, 2009; Dallel et al., 2018; Drummond et al., 2002; Griswold & Hogarth, 2018; Gu et al., 2015; Martinot et al., 2017; Pepling, 2012). Females produce ecdysone in response to mating and dietary cues, and the ovary is the predominant source of systemic ecdysone in adults (Ahmed et al., 2020; Ameku & Niwa, 2016; Ameku et al., 2017; Harshman, Loeb, & Johnson, 1999; Mirth et al., 2019; Sieber & Spradling, 2015). In the absence of ecdysone or EcR, oogenesis arrests at four critical developmental processes: germline stem cell (GSC) function, germ cell proliferation, follicle survival, and yolk uptake (Fig. 1D). Here, we highlight the lines of evidence suggesting that *Drosophila* oogenesis is regulated not only by ecdysone signaling, but by a larger network of nutritionally regulated NRs working in both ovarian cells and in peripheral tissues to stimulate oocyte production in response to nutrient availability.

5.1 GSC maintenance and proliferation are both directly and indirectly controlled by EcR signaling

Prior to adulthood, nutritional input guides ovary development and germline stem cell establishment. During early larval development, EcR and Usp repress the differentiation of primordial germ cells and somatic gonadal precursor cells, permitting their proliferation (Gancz, Lengil, & Gilboa, 2011; Hitrik et al., 2016). Later, pulses of ecdysone (likely from the prothoracic gland) promote EcR and Usp activation, providing a timing cue for the formation of stacks of gonadal somatic precursor cells. These so-called terminal filaments will become individual ovarioles in adults (Gancz et al., 2011; Hodin & Riddiford, 1998; Mendes & Mirth, 2016). EcR also promotes allocation of primordial germ cells into nascent somatic niches within the terminal filaments, thereby establishing the number of GSCs in each ovariole at eclosion (Gancz et al., 2011; König, Yatsenko, Weiss, & Shcherbata, 2011; Yatsenko & Shcherbata, 2018). The EcR co-activator Tai is likely involved in larval/pupal ovary development, as loss of *tai* leads to expanded numbers of cap cells and GSCs in newly eclosed females (König et al., 2011). Although most of the targets of EcR/Usp have not been identified in the developing gonad, the ecdysone early gene *br* is necessary for the effects of EcR signaling in this context (Gancz et al., 2011; Hitrik et al., 2016). Additionally, the *Drosophila* NR encoded by *E78* (most similar to vertebrate REV-ERB receptors) is necessary in cap cells prior to adulthood to establish the proper number of GSCs (Ables, Bois, Garcia, & Drummond-Barbosa, 2015). Although other NRs have not been described in ovary development, recent annotation of gene expression profiles for all cell types in the developing ovary will likely aid future experiments geared toward understanding how NRs guide ovary development in response to nutritional cues (Slaidina, Banisch, Gupta, & Lehmann, 2020).

In adult females, EcR signaling is necessary in GSCs for their self-renewal and proliferation (Figs. 2 and 3). Mutants in which ecdysone production (such as the temperature sensitive *ecdysoneless* mutants) or ecdysone reception (such as loss of function of *EcR*) display rapid GSC loss upon switching to a restrictive temperature (Ables & Drummond-Barbosa, 2010; König et al., 2011; Morris & Spradling, 2012). Moreover, a pulse of ecdysone biosynthesis at mating promotes an initial surge of symmetric GSC division, resulting in an overall increased number of GSCs per ovariole (Ameku & Niwa, 2016). While the phenotypes resulting from global loss of ecdysone function are likely a cumulative effect of disrupted signaling in multiple ovarian or peripheral cell types, several lines of evidence suggest that ecdysone is required cell autonomously in the GSCs for self-renewal (Fig. 2) (Ables & Drummond-Barbosa, 2010; Ahmed et al., 2020; Ameku & Niwa, 2016; Ameku et al., 2017; König et al., 2011; Morris & Spradling, 2012; Sieber & Spradling, 2015). GSCs lacking functional *usp* or the early gene *E74* exhibit reduced proliferation and fail to self-renew, likely due to modulation of BMP signaling (Ables & Drummond-Barbosa, 2010; König et al., 2011). Ecdysone also functions with the chromatin remodeling factor ISWI/NURF, an EcR co-activator, to regulate GSC self-renewal, suggesting cell autonomous regulation of GSCs (Ables & Drummond-Barbosa, 2010; Badenhorst et al., 2005). Although *E74* is the only ecdysone early gene known to be required to promote GSC self-renewal and proliferation, other transcriptional targets are likely to promote these processes downstream of EcR (Ables, Hwang, Finger, Hinnant, & Drummond-Barbosa, 2016). Elucidating EcR/Usp and E74 transcriptional targets is a key future direction necessary for understanding how ecdysone directly modulates GSCs.

Ecdysone signaling also regulates GSC self-renewal non-autonomously via somatic escort cells and cap cells (Fig. 3). EcR, Usp, and Taiman are highly expressed in cap and escort cells, and ligand-binding reporters for EcR and Usp indicate that ecdysone signaling is active in these cells (König et al., 2011; Morris & Spradling, 2012). Ecdysone-responsive enhancers in several gene loci, including *E75*, *ftz-f1*, and *br* are also active in cap and escort cells, suggesting a complex signaling network guides escort cell function (McDonald et al., 2019). Loss of *EcR*, *usp*, or *E75* specifically in escort cells results in decreased GSC number (Morris & Spradling, 2012). In contrast, loss of *EcR* or *taiman* in cap cells expanded the number of cap cells, resulting in more GSCs (König et al., 2011). Since escort cells participate non-autonomously in germ cell differentiation by limiting the range of BMP signals (König & Shcherbata, 2015; Luo, Wang, Fan, Liu, & Cai, 2015; Mottier-Pavie, Palacios, Eliazer, Scoggin, & Buszczak, 2016), ecdysone signaling may modulate one of the many paracrine signaling ligands produced by escort cells. Two possible candidates may be Wnt/Wg and/or Epidermal Growth Factor Receptor (EGFR) signaling. In the absence of ecdysone signaling, GSCs do not properly receive BMP signals, EGFR activity is increased, and cell adhesion between germ cells and escort cells is altered (König & Shcherbata, 2015). It is unclear, however, whether these are direct or indirect effects of EcR transcription in escort cells. Additional experiments testing how ecdysone signaling modulates paracrine signals in escort cells are necessary to resolve the molecular mechanism of action.

5.2 Ecdysone signaling mediates germline differentiation, follicle formation and encapsulation

Following separation of the cystoblast from the GSC, the cystoblast progresses through four rounds of mitotic division forming an interconnected cyst, while simultaneously initiating oocyte selection and differentiation (Fig. 1B and D) (Hinnant et al., 2020). Concurrent with oocyte differentiation, individual cysts are packaged into discrete egg chambers, encapsulated by follicle cells. These processes are inextricably intertwined, and include molecular mechanisms maintaining the self-renewal and proliferation of FSCs and their immediate daughters (Rust & Nystul, 2020). A variety of experiments have suggested that ecdysone signaling impacts these processes, perhaps via molecular mechanisms independent of germ cell differentiation. First, loss of ecdysone ligand (*ecdysoneless* mutants) results in fewer dividing cysts and fewer 16-cell cysts, indicating a block to germ cell differentiation (König et al., 2011; Morris & Spradling, 2012). Although inactivation of *E74* in germ cells blocks cyst division, in part due to increased apoptosis, *tai* depletion from escort cells causes a block in cyst differentiation and division, leading to excess single germ cells (Ables & Drummond-Barbosa, 2010; König et al., 2011). The EcR repressor Abrupt regulates this process through a feedback loop with ecdysone (Fig. 3) (König & Shcherbata, 2015; König et al., 2011). Abrupt blocks the ability of Tai to bind to EcR. The ecdysone responsive miRNA, *let-7*, targets *abrupt* transcripts, allowing Tai to bind EcR and increasing ecdysone signaling strength (König & Shcherbata, 2015). Unlike *bam* mutants, which completely block differentiation, loss of EcR signaling leads to a delay of differentiation, accompanied by a change in chromatin state (König et al., 2011; Ohlstein & McKearin, 1997). Ecdysone mutants lack the monoubiquitination of the histone H2B (H2Bub1) modification, which is required for the change from a GSC state to a differentiation state (Karpiuk et al., 2012; König & Shcherbata, 2015). These cells become temporarily stuck between GSC and cystoblast fates, indicating that ecdysone signaling is required in somatic cells for the committed germ cell differentiation fate.

Loss of ecdysone signaling in escort cells also abrogates cyst formation and encapsulation (Ables & Drummond-Barbosa, 2010; König et al., 2011; Morris & Spradling, 2012). Knock-down of *usp, EcR*, or *E75*, or overexpression of the EcR repressor *Abrupt*, in escort cells and follicle cells resulted in abnormally shaped escort cells and a decrease or absence of membrane extensions (König & Shcherbata, 2015; König et al., 2011; Morris & Spradling, 2012). It is unclear, however, exactly how ecdysone signaling modulates escort cell shape and function, and whether and how this impacts EGFR signaling. Given the unique spatiotemporal specificity of ecdysone signaling, it is also formally possible that ecdysone signaling promotes unique cell activities in posterior escort cells, FSCs, and pre-follicle cells (Fig. 3) (Ables et al., 2016). This could be due to unique combinations of EcR transcriptional targets, or perhaps due to differential availability of the ecdysone ligand. Indeed, knock-down of the ecdysteroidogenic enzymes encoded by *neverland*, *diembodied*, or *spook* in escort cells (under the control of the *Gal4* driver *c587-Gal4*), is sufficient to block the initial surge of ecdysone production following mating and steroid-dependent midgut growth (Ahmed et al., 2020; Ameku & Niwa, 2016). These results warrant new investigation as to which ovarian cells produce and import ecdysone. Recent characterization of specific reagents for *UAS/Gal4*-mediated CRISPR and RNAi, and ovarian cell

transcriptomic signatures, may help distinguish potential roles of ecdysone signaling in these somatic cell types (Hartman et al., 2015; Huang, Sahai-Hernandez, et al., 2014; Jevitt et al., 2020; McDonald et al., 2019; Port et al., 2020; Slaidina et al., 2020).

5.3 Ecdysone is required for continued egg chamber development, survival, and vitellogenesis during mid- and late-stages of oogenesis

The first observed phenotype associated with ecdysone mutants was the loss of vitellogenic egg chambers (Audit-Lamour & Busson, 1981; Buszczak et al., 1999; Carney & Bender, 2000). The few eggs that were laid by females had very thin eggshells with misshapen appendages (Audit-Lamour & Busson, 1981; Hackney, Pucci, Naes, & Dobens, 2007; Oro, McKeown, & Evans, 1992). While injection of ecdysone lead to loss of vitellogenic egg chambers, reduction of ecdysone signaling also abrogated egg chamber development, suggesting that the amount of ecdysone is critical for vitellogenesis. These phenotypes foreshadowed a variety of molecular mechanisms by which ecdysone signaling promotes continued oocyte development outside of the germarium.

After cysts are fully encapsulated, they move outside the germarium as individual egg chambers (Fig. 1A and D). As egg chambers pinch away from the germarium, follicle cells differentiate into stalk cells, pole cells, and main body follicle cells through Notch/Delta and Jak/Stat signaling (Duhart et al., 2017; Osterfield et al., 2017). This establishes egg chamber polarity and subsequent oocyte polarity as the oocyte continues to grow. During vitellogenesis, follicle cells proliferate, grow in size, differentiate, and migrate to specific locations around the oocyte to form the eggshell and exterior structures of the egg chamber, including the micropyle (which allows for sperm to enter the egg), dorsal appendages (which allow for gas exchange), and the operculum (the region from which the larvae emerges at hatching, post-fertilization). Concurrently, the oocyte is loaded with yolk proteins and lipids, especially triacylglycerol (Parra-Peralbo & Culi, 2011; Sieber & Spradling, 2015; Ziegler & Van Antwerpen, 2006). Because oogenesis is closely tied to nutritional status of the female fly, developing egg chambers must bypass a nutritional checkpoint at stage 8 prior to the switch to vitellogenesis. This either allows the egg chamber to proceed to undergo the metabolically demanding process of vitellogenesis or breakdown if nutrients are not favorable for subsequent embryo development (Peterson, Timmons, Mondragon, & McCall, 2015). Insufficient nutrients cause apoptosis and reabsorption of middle stage egg chambers (8–13) and withholding of stage 14 follicles, blocking ovulation.

EcR and several ecdysone responsive genes are highly expressed in both germline and somatic cells during the vitellogenic stages of oocyte development (Ables et al., 2016; Buszczak et al., 1999; McDonald et al., 2019). Loss of *EcR*, *E74*, *E75*, or *E78* all result in egg chamber death, suggesting important roles for ecdysone signaling during early vitellogenesis (Ables et al., 2015; Ables & Drummond-Barbosa, 2010; Ables et al., 2016; Audit-Lamour & Busson, 1981; Buszczak et al., 1999; Carney & Bender, 2000). Ecdysone levels are also the highest during vitellogenic stages, and several ecdysone biosynthesis genes are expressed (Buszczak et al., 1999; Ono et al., 2006; Petryk et al., 2003; Uryu et al., 2015). Interestingly, injection of 20E leads to increased apoptosis of nurse cells in stage 8 follicles and loss of all vitellogenic stages, indicating that the amount of ecdysone is very

important for regulation of apoptosis (Soller, Bownes, & Kubli, 1999; Terashima et al., 2005). The ecdysone early genes *br* and *E75* appear to regulate this nutritional checkpoint. When females are nutritionally deprived, *br* isoforms *Z2* and *Z3* are up-regulated, leading to expression of *E75A* which induces apoptosis of cysts, while simultaneously suppressing *E75B*, which is required to inhibit apoptosis (Terashima & Bownes, 2006).

Impairment of EcR and Usp signaling specifically in follicle cells during vitellogenesis results in reduced egg size, thinner eggshell, and malformed dorsal appendages and operculum (Hackney et al., 2007). These phenotypes can be attributed to a variety of molecular mechanisms. First, EcR-B1 in follicle cells promotes epithelial integrity, microvilli formation, and follicle cell shape (Fig. 4) (Hackney et al., 2007; Romani et al., 2009; Romani, Gargiulo, & Cavaliere, 2016). EcR-B1 regulates expression of adherens and basolateral junction proteins to maintain the connections between follicle cells, and between the follicle cells and germ cells (Fig. 4) (Romani et al., 2009). EcR also promotes follicle cell endocycling and expression of eggshell proteins (Bernardi, Romani, Tzertzinis, Gargiulo, & Cavaliere, 2009; Sun, Smith, Armento, & Deng, 2008). Second, follicle cell migration is driven by the interplay of EcR signaling with Jak/Stat signaling (Fig. 5) (Peercy & Starz-Gaiano, 2020). In this context, cell migration occurs in direct response to a pulse of ecdysone synthesis, which dislodges the ecdysone co-repressor Abrupt (Bai et al., 2000; Buszczak et al., 1999; Jang, Chang, Bai, & Montell, 2009). This allows EcR-A, EcR-B, and Usp to be activated by Tai in border cells, which promotes turnover of the cell adhesion proteins E-cadherin and β-catenin (Bai et al., 2000; Jang et al., 2009) and delamination from the follicle cell epithelium. Border cells then continue to migrate through the nurse cells, sending out projections until they reach the anterior border of the oocyte at stage 10 (Peercy & Starz-Gaiano, 2020). Overexpression of ecdysone signaling leads to early migration of border cells while decreased ecdysone delays migration, indicating that ecdysone helps to regulate timing (Bai et al., 2000; Cherbas et al., 2003; Domanitskaya, Anllo, & Schüpbach, 2014; Jang et al., 2009). At least four other NRs (E75, Hr3, Hr4, and Ftz-f1) also regulate border cell migration (Manning et al., 2017; McDonald et al., 2019; Wang et al., 2020). It is unclear however, whether these NRs functions independently of EcR/Usp, or together in an elaborate transcriptional network. Lastly, Usp and Br are required for dorsal appendage formation and amplification of chorionic genes (Deng & Bownes, 1997; Oro et al., 1992; Osterfield et al., 2017; Tzolovsky, Deng, Schlitt, & Bownes, 1999). In stage 6, Br is expressed in all follicle cells but becomes progressively restricted to two groups of dorsal-lateral-anterior follicle cells that become the cells of the dorsal appendage. Ecdysone signaling and the NR Ftz-f1 are also required for ovulation and reproductive tract development (Knapp, Li, Singh, & Sun, 2020; Knapp & Sun, 2017; Sun & Spradling, 2012).

6. Signaling from peripheral tissues promotes GSC maintenance

Although NRs acting in the ovary are crucial for fertility, a developing area of interest in the field is how NRs located in other tissues can influence oogenesis. Reproduction is energetically costly to females; thus, the maternal metabolic physiology must be tailored to turn specific dietary nutrients into the lipids and proteins necessary to sustain egg development (Armstrong, 2020; Mirth et al., 2019; Sieber & Spradling, 2017). As in humans, maintenance of a maternal physiology capable of sustaining egg production relies

on several interrelated organs, including the brain, the fat body (analogous to human liver), and the gut (Droujinine & Perrimon, 2016; Rajan & Perrimon, 2011). Recent studies reveal that a complex interorgan communication network not only links these organs to oogenesis, but also utilize ecdysone produced from the ovary to modulate organ maintenance and function (Ahmed et al., 2020; Ameku & Niwa, 2016; Matsuoka, Armstrong, Sampson, Laws, & Drummond-Barbosa, 2017; Sieber & Spradling, 2015; Weaver & Drummond-Barbosa, 2019).

The fat body, an organ which helps to maintain maternal metabolism, is particularly appealing as a candidate tissue that influences oogenesis. The fat body is composed of adult adipocytes, which store lipids, and oenocytes, the hepatocyte-like cells of insects that produce lipids and other macromolecules (Armstrong, 2020; Arrese & Soulages, 2010). Yolk proteins and the yolk storage protein vitellogenin are produced by the fat body and transported to the oocyte; both of these processes are influenced by ecdysone signaling (Gilbert, Serafin, Watkins, & Richard, 1998; Jowett & Postlethwait, 1980; Schonbaum, Perrino, & Mahowald, 2000; Sieber & Spradling, 2015; Yan & Postlethwait, 1990). EcR promotes the female metabolic state by promoting high levels of whole-body triglycerides and glycogen, likely via effects on the fat body (Sieber & Spradling, 2015). The NR encoded by *seven up* (*svp*), most closely related to mammalian COUP-TFII, is also expressed in adipocytes and essential in adipocytes and oenocytes for ovarian GSC self-renewal and egg chamber survival, respectively (Weaver & Drummond-Barbosa, 2019). Intriguingly, other endocrine signals are also produced by the fat body that regulate oogenesis, notably insulin signaling (Armstrong & Drummond-Barbosa, 2018; Armstrong, Laws, & Drummond-Barbosa, 2014; Matsuoka et al., 2017). These results suggest that peripherally located NRs may act in concert with other endocrine signaling pathways to fine-tune oogenesis in response to maternal diet.

7. Conclusions and open questions

NRs are key regulators of many aspects of development, reproduction, and metabolism. A comprehensive understanding of NR target genes, signaling mechanisms in and between cell types, and communication between organs can provide insight into possible conserved mechanisms between *Drosophila* and humans. Yet many questions remain for future studies. First, what are the specific transcriptional targets of ecdysone signaling in oogenesis, and how are these regulated in a precise spatiotemporal manner? Although ecdysone-mediated processes are well-understood, we are only just beginning to understand how ecdysone biosynthesis, transport, and reception by EcR are achieved in ovarian cells. Addressing this complex question will require sophisticated genetic manipulation in cell-specific studies. Recent transcriptomic profiling of ovarian cells by single cell RNA sequencing may be a tremendous step forward in this area (Jevitt et al., 2020). Second, how does ecdysone signaling promote egg chamber viability and progression into vitellogenesis? Several ecdysone-responsive NRs are clearly expressed in nurse cells during these stages and required for egg chamber survival, but it is unclear whether they may promote an as-yet undescribed nutritional checkpoint in early oogenesis or promote cyst growth. Lastly, are roles of EcR or other NRs unique to female reproduction? Although the role of ecdysone in germline stem cell maintenance has been most commonly associated with oogenesis, recent

studies have demonstrated a potential contribution of ecdysone to spermatogenesis in *Drosophila* (Li, Ma, Cherry, & Matunis, 2014). The effects of obesity, metabolic dysfunction, and diabetes on reproductive tissues are not unique to females, and NRs also regulate many aspects of spermatogenesis in both *Drosophila* and humans. Future studies analyzing the role of nutritionally regulated NRs in spermatogenesis are also warranted.

Determining the mechanisms by which hormone signaling facilitates crosstalk between germline and somatic cells could provide valuable insight into possible conserved mechanisms with other species, such as humans. In *Drosophila*, signals from the germline greatly influence follicle cell function and vice versa. It has been well-established that ecdysone signaling in the escort cells influences GSC self-renewal, germ cell differentiation, and follicle cell encapsulation and movement. It is less clear, however, whether ecdysone signaling in germ cells might influence the overlying somatic tissue. Given the parallels between steroid hormone signaling in *Drosophila* and humans, future studies investigating how ecdysone facilitates germline-soma communication could provide a better understanding of the mechanisms of hormone signaling in human oogenesis.

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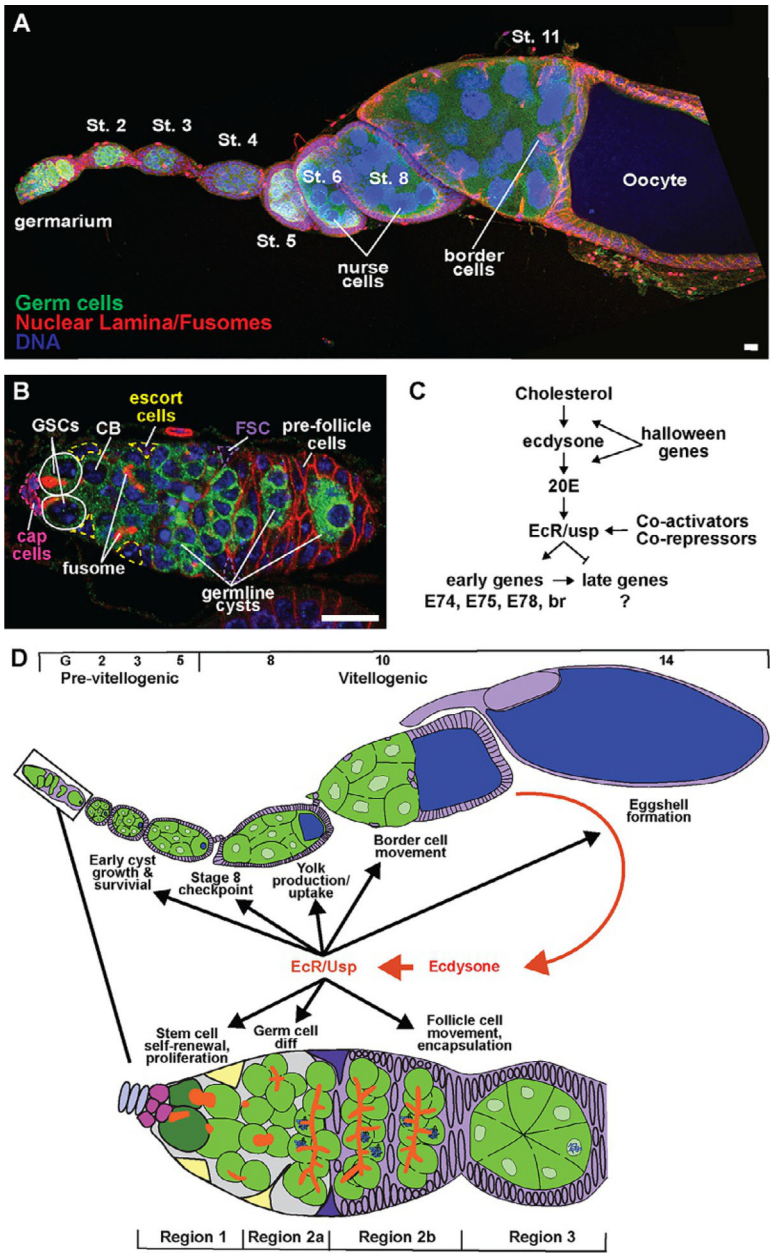

Fig. 1.

Ecdysone signaling regulates *Drosophila melanogaster* oogenesis. (A) Maximum intensity projection image of a *Drosophila* ovariole, labeled with anti-Vasa (green; germ cells), anti-Hts (red; fusomes and follicle cell membranes), anti-LamC (red; nuclear envelope of cap cells) and DAPI (blue, DNA). (B) Optical cross section of the *Drosophila* germarium showing GSCs (solid white line) anchored to cap cells (dashed pink line), which make up the GSC niche. Germ cells are characterized by the presence of a fusome (orange), which extends as germ cells divide. Escort cells (yellow dashed line) signal to GSCs to promote differentiation. Follicle stem cells (FSC; purple dashed line) create pre-follicle cells that surround the 16-cell cyst, giving rise to an egg chamber or follicle that leaves the

germarium. (C) Schematic of the ecdysone signaling pathway. (D) Summary of ecdysone-regulated processes in the ovariole and germarium. Scale bar = 10μm.

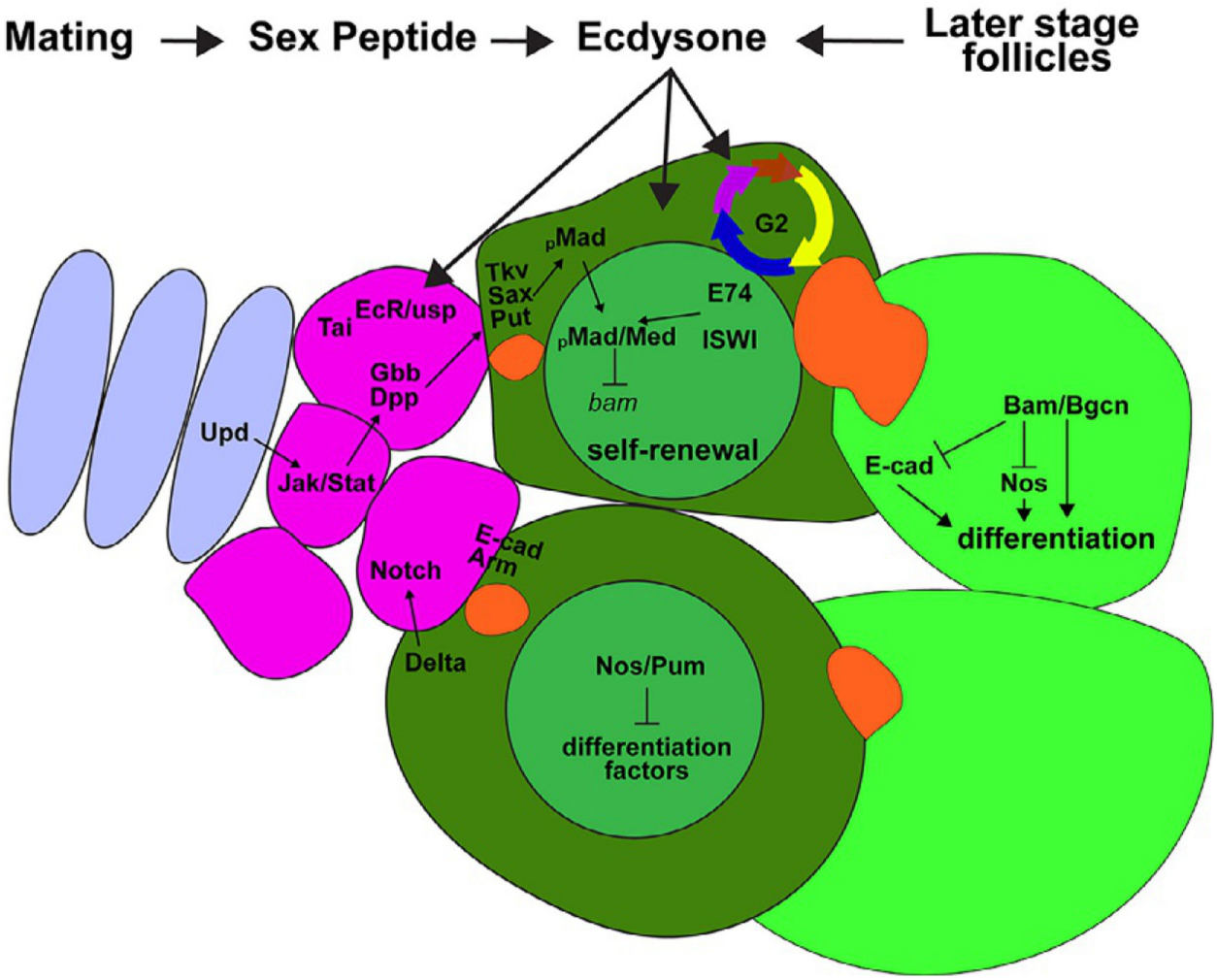

Fig. 2.

Signaling pathways that intrinsically regulate GSC self-renewal and cystoblast differentiation. Ecdysone levels are increased due to sex peptide, which is deposited during mating and made in later stage follicles. Ecdysone is received in cap cells (pink) and GSCs (dark green). Terminal filament cells (purple) secrete the ligand Unpaired (Upd) to cap cells to stimulate the Jak/Stat pathway. The Jak/Stat pathway and ecdysone signaling regulate BMP signaling. EcR/Usp binds to Tai to control cap cell function. EcR regulates the BMP signaling pathway to regulate GSC self-renewal. The BMP ligands Gbb and Dpp are secreted from cap cells to the receptors on GSCs. This phosphorylates Mad, which will dimerize with Med and repress bam transcription. Ecdysone targets are intrinsically required to maintain GSC self-renewal. In differentiating CBs (light green) bam is transcribed and represses E-cadherin and Nos thereby committing the cell to differentiation.

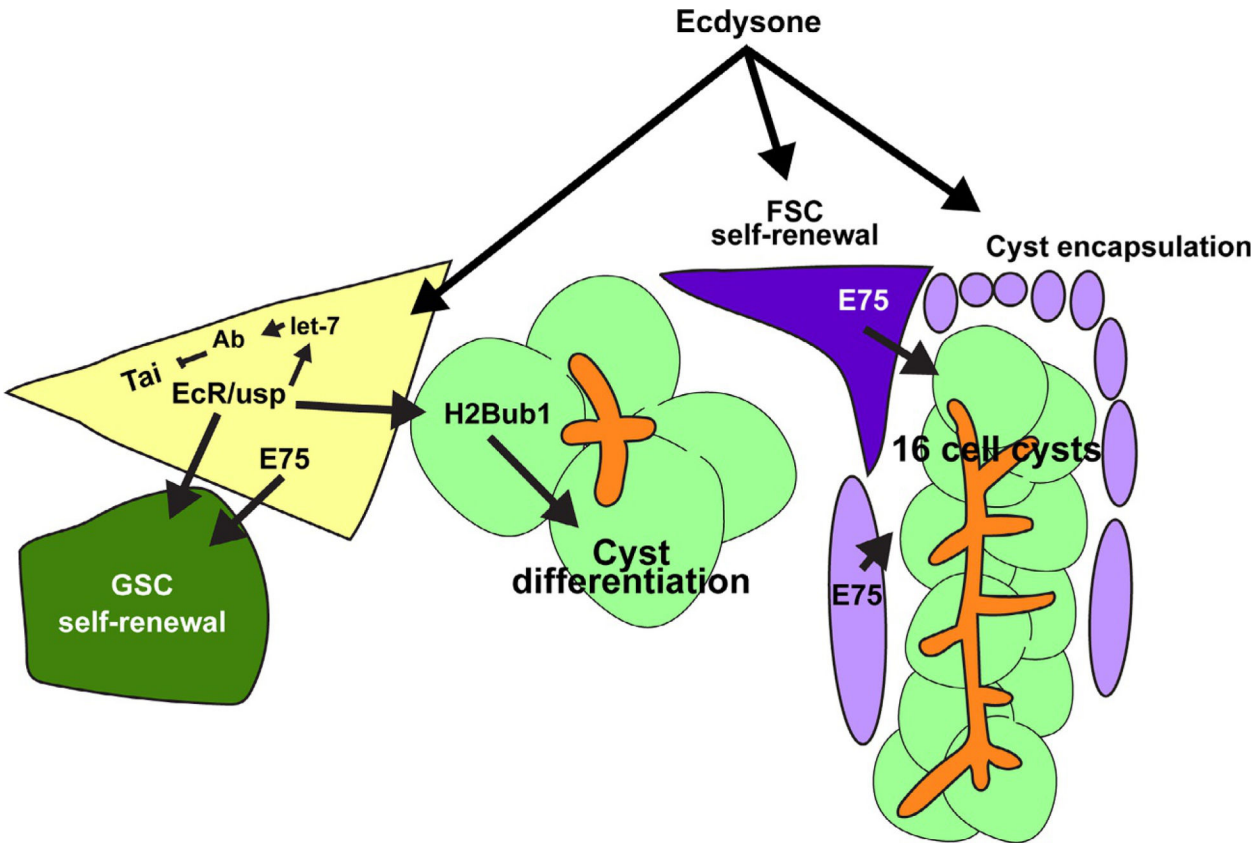

Fig. 3.

Ecdysone is required in somatic cells for early germline processes. In the escort cells (yellow), EcR binds to the co-activator Tai to regulate GSC (dark green) self-renewal. Ecdysone functions in a feedback loop with the miRNA let-7 and the repressor Abrupt. Ecdysone regulates the monoubiquitination of the histone H2B (H2Bub1) modification which allows for cysts (light green) to differentiate. Ecdysone signaling in the FSCs (dark purple) and follicle cells (light purple) drives formation and encapsulation of 16 cell cysts.

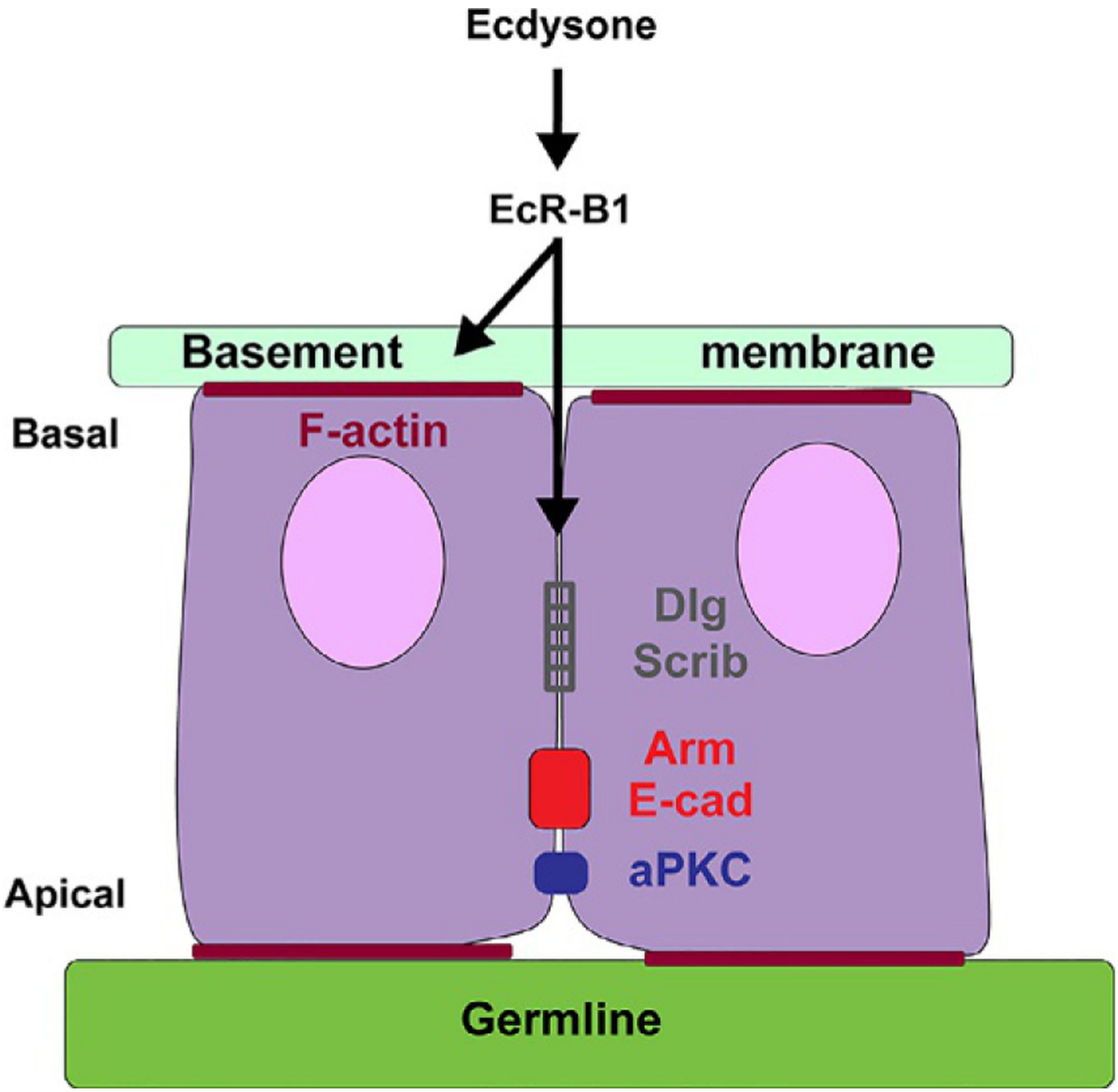

Fig. 4.

Follicle cell polarity in mid-stages is driven by ecdysone signaling. EcR-B1 regulates F-actin (maroon) expression, and localization of the adherens junction proteins Arm and E-Cad (red), the septate junction proteins Dlg and Scrib (gray), and aPKC (blue), in mid-stage follicle cells (purple).

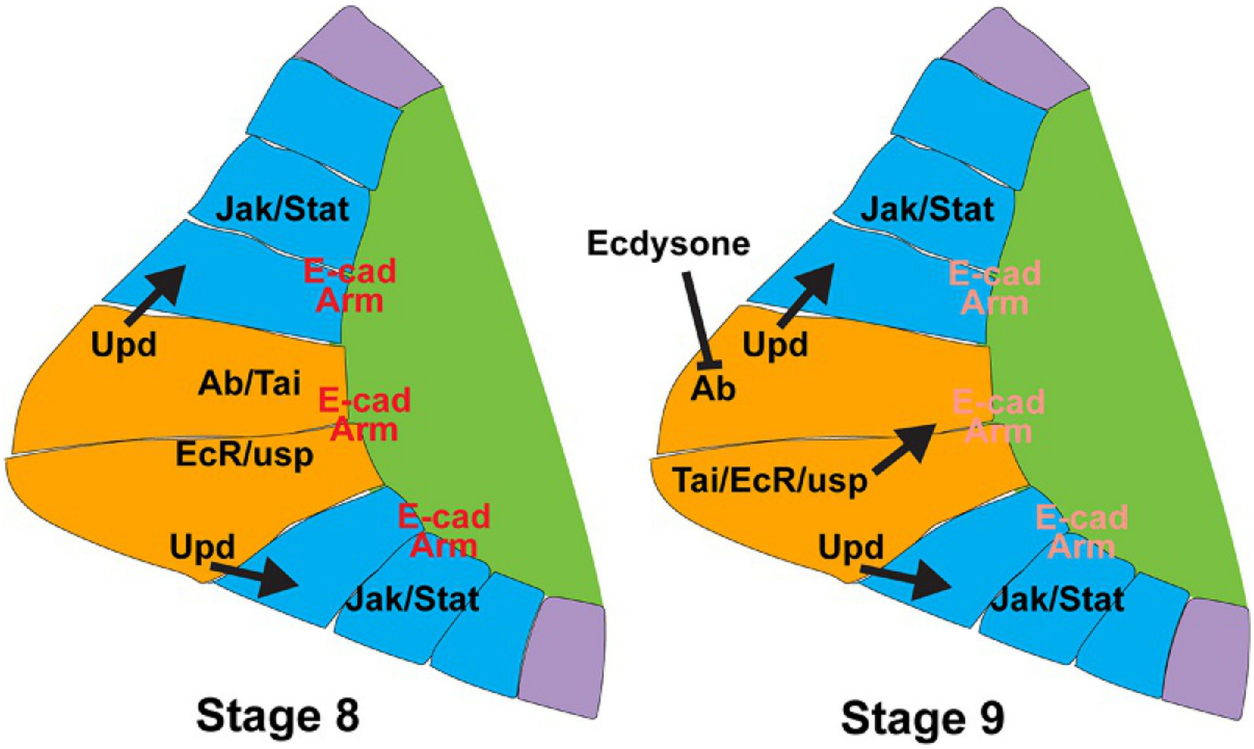

Fig. 5.

Ecdysone signaling regulates the timing of border cell movement. Upd ligand in polar cells (orange) establishes future border cells (blue) from follicle cells (light purple). In stage 8 follicles, Abrupt binds Tai and prevents its binding to EcR. This blocks turnover of the cohesion proteins E-cadherin and β-catenin (arm). In stage 9 border cells, ecdysone inhibits Abrupt, allowing Tai to bind to EcR. Tai/EcR creates turnover of E-cadherin and β-catenin, promoting border cell migration.