INVESTIGATING THE ROLE OF ECDYSONE RECEPTOR IN GERMLINE TO SOMATIC CELL COMMUNICATION

by

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Investigating the Role of Ecdysone Receptor in Germline to Somatic Cell Communication

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Abstract

Oogenesis requires complex coordination between both somatic and germ cell populations, relying on systemic signals such as hormones for proper oocyte production and formation. *Drosophila melanogaster* is an excellent model organism to explore how signaling from germ to somatic cells is required for viable egg production. *Drosophila* oogenesis utilizes steroid hormones such as ecdysone to facilitate many aspects of germ cell development such as ovariole formation, germline stem cell proliferation, and cyst formation**.** Ecdysone function relies on reception by the heterodimeric complex, Ecdysone Receptor (EcR) and Ultaspiracle (Usp), that in turn activates transcriptional targets. EcR and Usp are expressed in both germline and somatic cell populations, but the role that ecdysone signaling plays in germ cells to control somatic cell processes is unclear. To allow germline compatibility, three EcR RNAi lines and three dominant negative lines were developed in *Drosophila* to deplete or block EcR function in the germline. Using various germline specific drivers, we found that loss of EcR results in significant somatic cell focused phenotypes. Irregularities such as non-ascending egg chambers where egg chambers stall in repeating stages were observed. Upon further phenotypic analysis, we also discovered cyst collision phenotypes where 16-cell cysts were not properly encapsulated by somatic follicle cells. There was also a significant decrease in follicle cell proliferation, suggesting that EcR is needed to stimulate proliferation of overlying somatic cells. Overall, this suggests that EcR is needed in the germline for follicle assembly. We plan to further investigate germ to somatic cell communication using live imaging to visualize both follicle cell migration. This will inform us if EcR non-autonomously promotes follicle cell migration through centripetal movements.

Acknowledgments

I would like to sincerely thank the people and organizations that made this project possible. First and foremost, I am extremely grateful to my advisor, Dr. Elizabeth Ables for her invaluable mentorship, guidance, and immense knowledge she has gifted me. I would not be where I am without her support, and I am forever thankful for all the hours she has given me. This project would not be possible without the help of Ables Lab members. A special thanks to lab members Amanda Powell and Lauren Jung for their friendship, encouragement, and feedback. I would also like to thank Dr. Lauren Anllo for her insightful comments and advisement as well as her enthusiastic attitude. Finally, thank you to Developmental Studies Hybridoma Bank, Bloomington *Drosophila* Stock Center, and the Department of Biology for stocks and reagents. Funding was received from East Carolina University URCA awards, ECU Honors College, and the National Institute of Health (GM117502).

Table of Contents

List of Figures

INTRODUCTION

Many species undergo the process of oogenesis to create viable eggs necessary to seed the next generation. Within gamete-producing ovaries, germ cells mitotically expand and differentiate to form an oocyte while somatic cells aid in oocyte development and eggshell production. This process requires continuous coordination and bi-directional signaling from somatic cells to germ cells in order produce a viable egg (Pepling et al., 1999).

In mammals, oogenesis begins with differentiation of gamete producing cells (primordial germ cells), that divide mitotically to form oogonia. These germ cell clusters enter meiosis I, where they are termed oocytes, and they proceed through most of prophase before arresting at the diplotene stage (Alam & Miyano, 2020). At this point, oocytes become surrounded by a single layer of somatic, squamous-shaped pre-granulosa cells that characterize the primordial follicle unit. As the growth phase continues, pre-granulosa cells become cuboidal in shape and proliferate to form the primary follicles. Multiple layers of both granulosa and theca cells are generated to encapsulate the oocyte to become the secondary follicles (Clarke, 2018). Nearing its complete growth, the oocyte develops a fluid-filled cavity to separate granulosa cell types and continue meiotic maturation (Alam & Miyano, 2020). Through the release of numerous hormonal signals, the oocyte is triggered to ovulate where it can then be fertilized to form a viable zygote. One of the most complex steps of this process is the regulation of somatic cell populations in preparing the developing oocyte for encapsulation (Rios-Rojas et al., 2015). However, the extent of communication between these two cell populations is not well understood.

Drosophila melanogaster is a powerful experimental model used to study the mechanisms by which germ cells and the soma communicate during oogenesis. Each female *Drosophila* ovary is composed of 15-20 linear strings of developing oocytes termed ovarioles (Fig. 1 A-B). The germarium is located towards the anterior most tip and houses the germline stem cells (GSC) in a specialized stem cell niche (Fig. 1C-D). GSCs asymmetrically divide to produce a self-renewed germline stem cell and a cystoblast. The cystoblast continues to mitotically divide into a 2, 4, 8, and 16-cell cyst. Once GSC daughters leave the niche, they are encapsulated by somatic escort cells (H. Lin & Spradling, 1993). Beginning in Region 2A, precursor follicle cells centrally migrate and start to encapsulate a 16-cell cyst in somatic follicle cells (Figure 1D). This partitions off individual 16-cell cysts, forming an egg chamber. Within each egg chamber, one cell differentiates to become a posteriorly positioned oocyte, while the other 15 cells differentiate to become supporting nurse cells which dump their cytoplasm and nutrients into the oocyte (Hinnant et al., 2020).

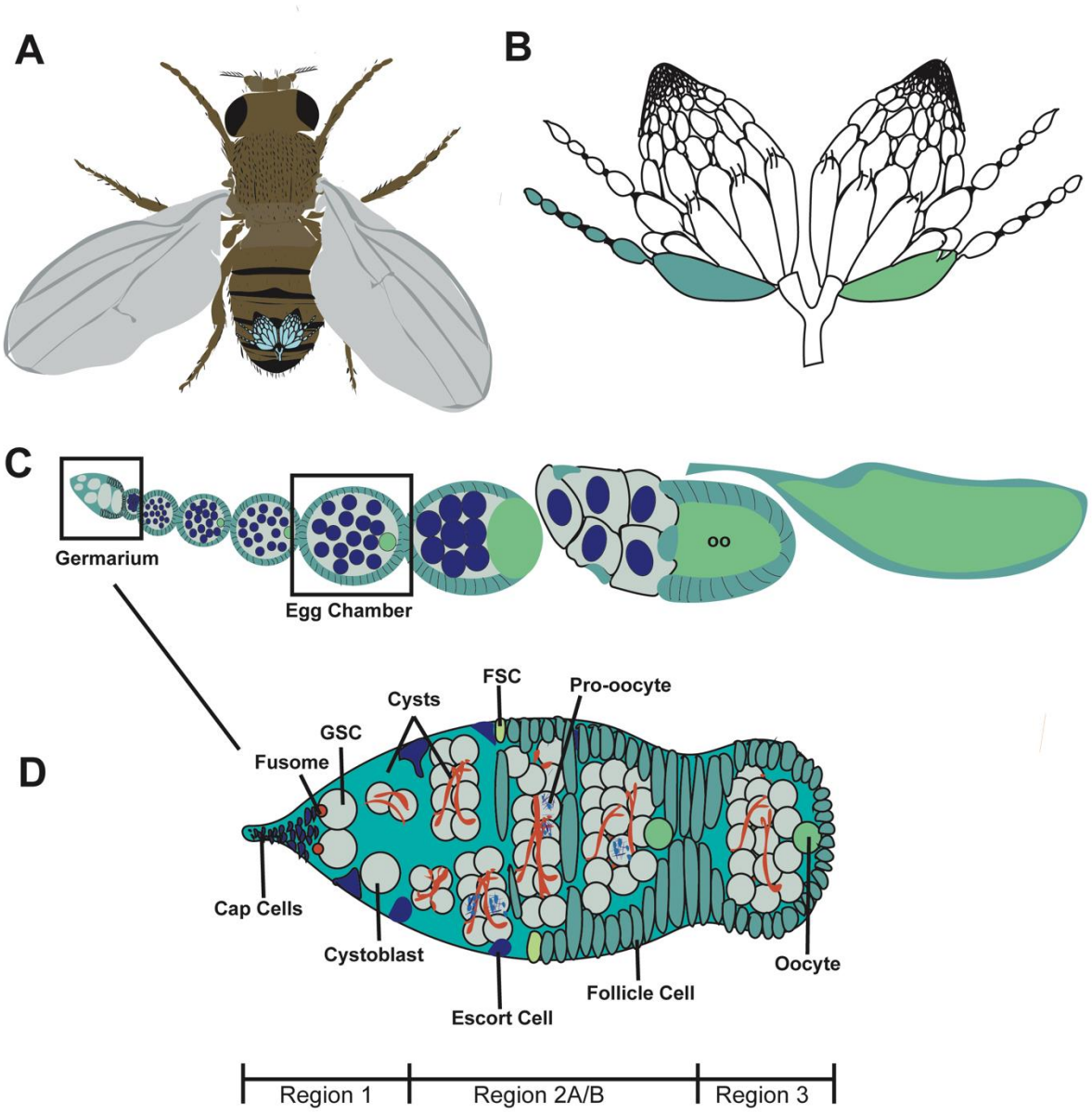


Figure 1: *Drosophila ovary offers a linear array to study oocyte development*

[A] A female *Drosophila* with a pair of ovaries (blue) [B] Enlarged ovaries showing both ovarioles (blue) and a developed egg (green). [C] An ovariole contains various stages of egg chambers, eventually producing an oocyte (green). The germarium is at the most anterior with egg chambers following. [D] Enlarged germarium with various cell populations. Cap cells (dark blue) reside in the most anterior tip and form the stem cell niche with germ line stem cells (GSC's, light blue) identified by their round fusome (red). GSCs asymmetrically divide to produce a cystoblast (light blue) which mitotically divides, producing 2, 4, 8, and 16 cell cysts surrounded by follicle cells (teal). Follicle cells encapsulate cysts to contain 15 nurse cells and one oocyte (green) in region 3.

In addition to signaling pathways, a variety of systemic signals like steroid hormones are important in regulating oocyte development. In *Drosophila*, the steroid hormone ecdysone helps facilitate oogenesis by promoting GSC division and maintenance (Ables & Drummond-Barbosa, 2010). Ecdysone signals act via reception by a nuclear hormone receptor protein Ecdysone Receptor (EcR) which forms a heterodimeric complex with Ultraspiracle (Usp) to activate transcriptional targets such as *E74, E75, and Br* (Figure 2A**)** (Hu et al., 2003; Koelle et al., 1991). Within the soma, ecdysone signaling has been shown to autonomously impact follicle growth and development (Buszczak et al., 1999) in addition to regulating various subpopulations of follicle cells such as the migration of border cells (Ables et al., 2015; Bai et al., 2000; Jang et al., 2009; Romani et al., 2009). Produced by late-stage ovarian follicles, ecdysone indirectly promotes 16-cell cyst production as well as cyst encapsulation by somatic escort cells (Morris & Spradling, 2012). While not debated in follicle cells, ecdysone's role in the germline is not as well understood. Data suggests ecdysone regulates maintenance and proliferation of GSC populations as well as egg chamber maturation and cell cycle defects (Ables et al., 2015; Buszczak et al., 1999), however its specific purpose in the germ cells has not been uncovered.

In this study, I wanted to investigate what happens when EcR is lost from the germline. Loss of EcR using three germline-specific RNA interface (RNAi) lines to block EcR mRNA [EcR RNAi 6-4, 2-11, 1-8] as well as 3 dominate negative lines [EcR F645A, W650A, DBL] to block EcR transcriptional activation caused variety of interesting phenotypes. At first, I hypothesized that depletion of EcR would result in egg chamber death. Instead, I notice a lack of ascending egg chambers, leading me to believe EcR plays a role in cyst progression. Additionally, I find that depleted EcR leads to severe germline cyst collision issues, characterized by decreased region 2B-3 distances. Finally, I propose two mechanistic models to address the germline specific role of EcR in promoting follicle cell proliferation and migration. Overall, these data suggest that in addition to its well-characterized roles in somatic follicle cells, EcR is necessary in the germline to promote timely egg chamber assembly and development

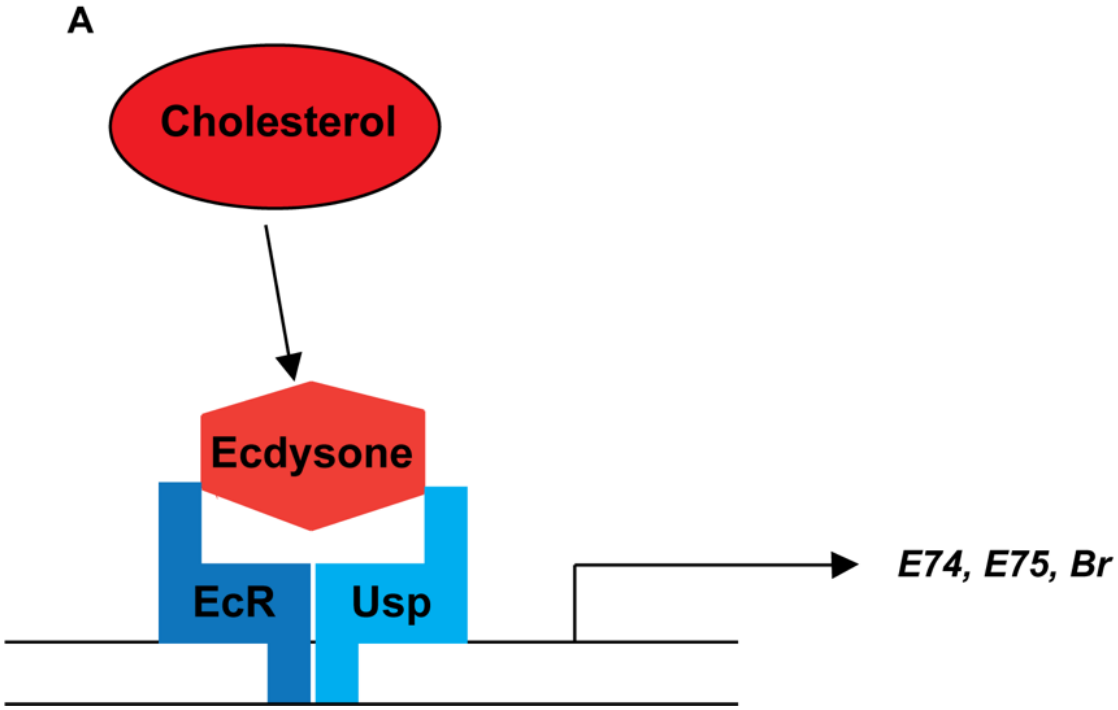


Figure 2: *Ecdysone signaling pathway in Drosophila*

[A] Ecdysone signaling is derived from dietary cholesterol. Signaling in response to the hormone relies on reception by a nuclear hormone receptor Ecdysone Receptor, forming a heterodimeric complex with Ultraspiracle.

RESULTS

Loss of EcR in the germline does not result in significant egg chamber death

Loss of EcR or its transcriptional target genes in the germline inhibits cysts from forming properly, demonstrating that ecdysone signaling is needed for egg chamber growth and survival (Buszczak, 1999). However, prior studies did not address the mechanism by which EcR promotes egg chamber development. I hypothesized that loss of EcR in the germline induces egg chamber death. I tested EcR-RNAi 6-4, one of three RNAi lines, by driving knockdown in all germ cells using the *nanosGal4::VP16* driver. Flies were dissected 6 days after eclosion (DAE). Ovaries were then stained for *Drosophila* cleaved caspase (DCP1), a caspase involved in programmed cell death, to mark all cells undergoing apoptosis as well as Hts and LamC. DAPI was added to visualize cell nuclei. DCP1 positive cells were observed per egg chamber and analyzed as a percentage. Although EcR-RNAi 6-4 yielded 18% DCP1 positive egg chambers, control cysts also showed 17%, making it non-significant (Figure 3A-C). This data suggests that EcR does not control egg chamber survival.

Loss of EcR in the germline slows egg chamber growth

I next wanted to investigate if loss of EcR results in disrupted egg chamber formation, unrelated to an apoptotic death. This question was previously addressed by inducing germline mutations in the EcR early target gene *E75,* which caused egg chamber arrest in stages 6 and 7 (Buszczak et al., 1999). Additionally, it has been shown that reducing specific growth-promoting factors such as Target of rapamycin (TOR), a nutrient sensing-pathway, in the *Drosophila* ovary results in a slowed egg chamber progression as well as follicle cell death (LaFever et al., 2010). Oogenesis in *Drosophila* encompasses 14 developmental stages ((Buszczak et al., 1999). A typical ovariole contains 3-4 pre-vitellogenic egg chambers, with the youngest and smallest on the anterior of the chain of progressively larger egg chambers.

To test if EcR is involved in cyst growth, I used *EcR-RNAi 6-4* to knock down EcR mRNA in all germ cells using the *nanosGal4::VP16* driver. 5 days after eclosion, flies were dissected and stained with Vasa to visualize germ cells as well as Hts/LamC and DAPI. Images were quantified according to the materials and methods section titled *Analysis and Statistics* (Figure 4C). *UASp-lacZ* control flies always had egg chambers in ascending order with smaller, anterior stages progressing into larger stages. However, *EcR-RNAi 6-4* had egg chambers with repetitive stages in non-ascending orders. Only 80% of ovarioles were found to ascend properly in *EcR-RNAi 6-4* (Figure 4A-D). Interestingly, often only pre-vitellogenic stages 2 and 3 repeated. This suggests that loss of EcR in developing germ cells results in a delayed, stalled growth of egg chambers.

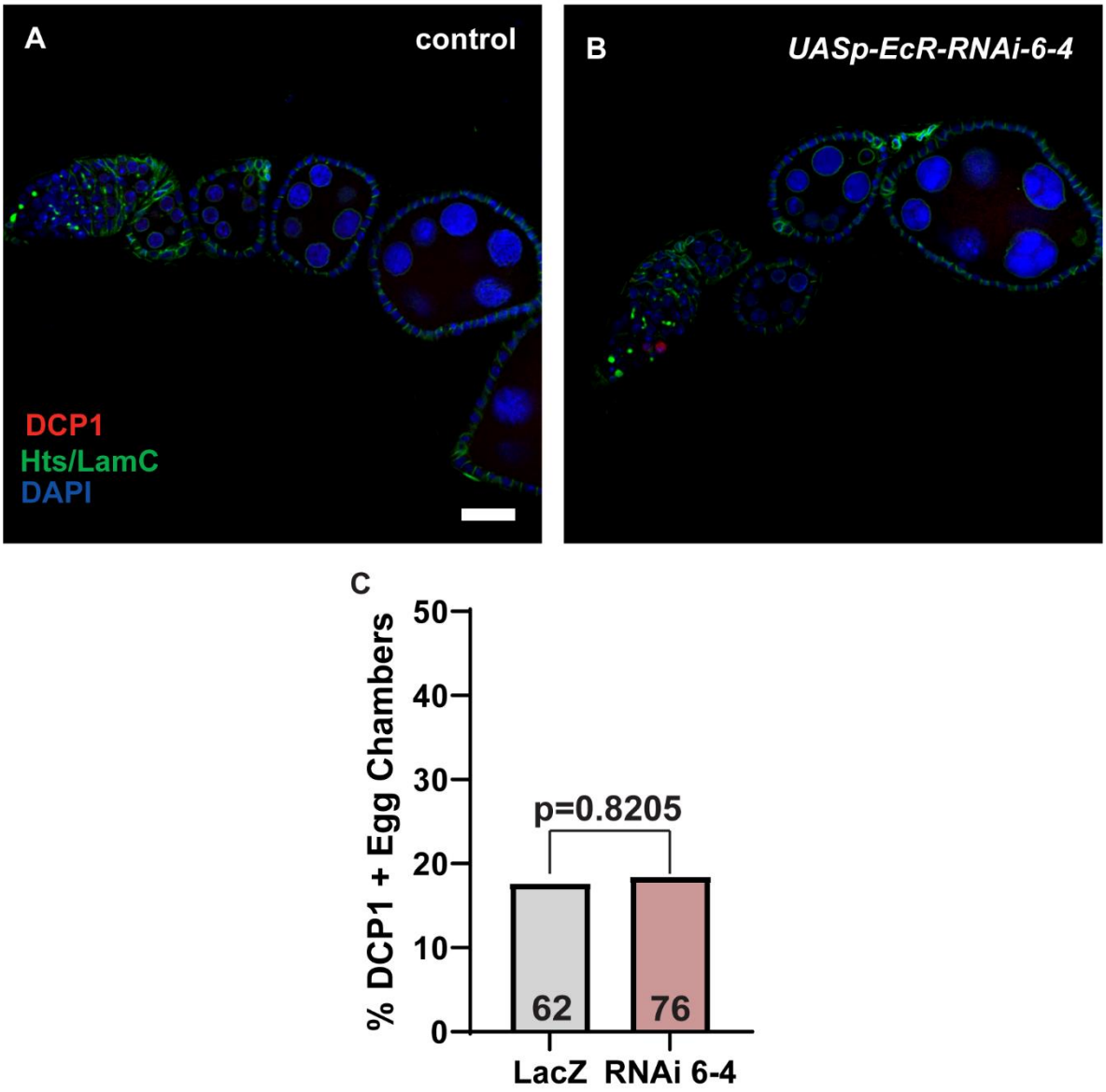


Figure 3: *EcR does not influence egg chamber survival*

[A-B] Ovarioles from females dissected 6 days after eclosion labeled with DCP1 (red), anti-Hts (green), anti-LamC (green), and DAPI (blue). *nanosGal4::VP16>UASp-lacZ control* [A] and *nanosGal4::VP16>UASp-EcR RNAi 6-4* [B]. [C] Percentage of DCP1 positive egg chambers found to be not significant. *P<0.01, Chi-Squares Test, scale bar 10 µm.

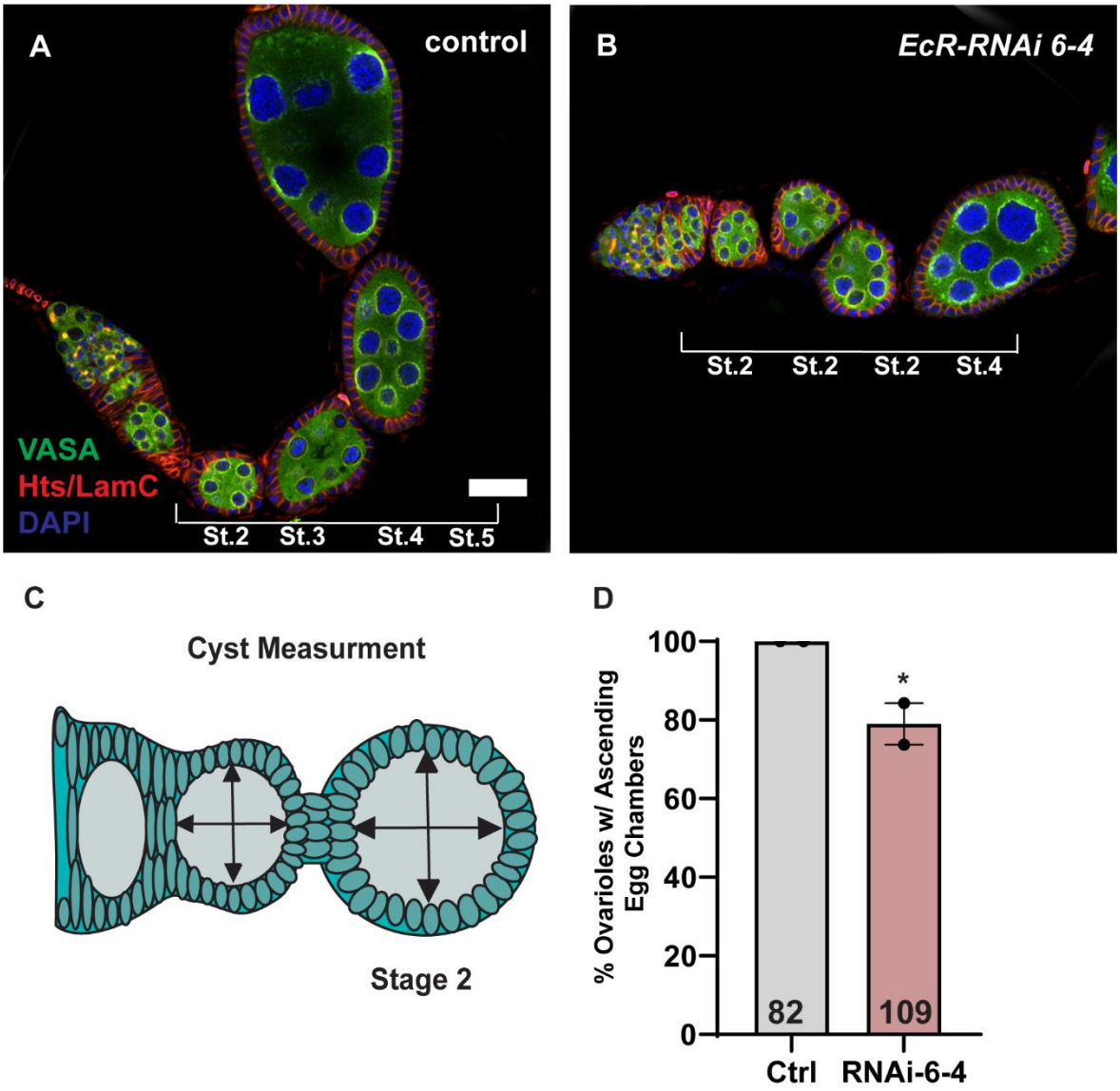


Figure 4: *EcR controls the growth of developing egg chambers*

[A-B] Ovarioles from females dissected 5 days after eclosion stained with Vasa (green), anti-Hts (red), anti-LamC (red), and DAPI (blue). *nanosGal4::VP16>UASp-lacZ control* [A] and *nanosGal4::VP16>UASp-EcR RNAi 6-4* [B]. [C] Schemat.ic diagram of how cyst measurements were performed. [D] The percentage of egg chambers that properly ascended in numerical order. Error bars show SEM. Students two tailed T-test, *P<0.01, scale bar 10 µm.

In order to limit what part of development these phenotypes were originating from, the same experiment was performed using *OtuGal4::VP16*. This driver still allows germline specificity, but it limits expression to 16 cell cysts and out, allowing GSCs and early cyst development to function as normal. This would answer the question of if the phenotype was stemming from an early disruption or from loss during mid-oogenesis. I used all three RNAi lines to distinguish if one had a more significant phenotype than the other. Egg chambers were dissected 5 days after eclosion (DAE) and stained with the germ cell marker Vasa, as well as Hts/LamC and DAPI. Cysts were quantified using the same methods as *nanosGal4::VP16* ovarioles. Consistent with knockdown throughout germ cell development, driving all three EcR RNAi lines with *Otu-Gal4* resulted in delayed growth when compared to control flies. Based on the expression pattern of *Otu-Gal4* , I conclude that the issue originates near region 2b/3 where the first 16 cell cyst is formed (Figure 5A-E). *EcR-RNAi 6-4* showed the most significant delayed growth phenotype, with egg chambers only ascending 60% of the time (Figure 5B, E).

I then wanted to see if I could recapitulate the same phenotype using another form of EcR knockdown. Following the method of Cherbas et. al., we constructed three inducible EcR transgenes carrying various point mutations (Cherbas et al., 2003). These included an overexpression of EcR termed *EcR-C655* which undergoes transcriptional activation consistent with wildtype EcR. *EcR-F645A* and *EcR-W650A* neither activate transcription in response to EcR ligand, acting as inhibitors of wildtype EcR. The final line, *EcR-Double* (DBL), contained both point mutations as found in *F645A* and *W650A.* Using the same germline specific drivers as above, I dissected, stained, and quantified ovarioles using the same techniques. When crossing the dominant negative and overexpression versions of EcR to *nanosGal4::VP16,* ovarioles presented tumorous germaria filled with undifferentiated cells and failed to make normal egg chambers; therefore, I was unable to quantify any effect on egg chamber progression (Jung, unpublished). However, when using *OtuGal4::VP16,* ovaries formed without tumors. Overexpression of EcR using the C655 line showed a similar phenotype to the control, suggesting it functions as wildtype EcR (Figure 6E-F). Consistent with the RNAi lines, overexpression of EcR dominant negative transgenes using *OtuGal4* also slowed egg chamber development, quantified by reduced incidence of ascending egg chambers. The phenotypes were most significant in *EcR-F645A* and *EcR-DBL* (Figure 6A-F). Overall, this data suggests that loss of EcR in the germline using various tools causes a delayed growth of egg chambers, making egg chambers appear as non-ascending.

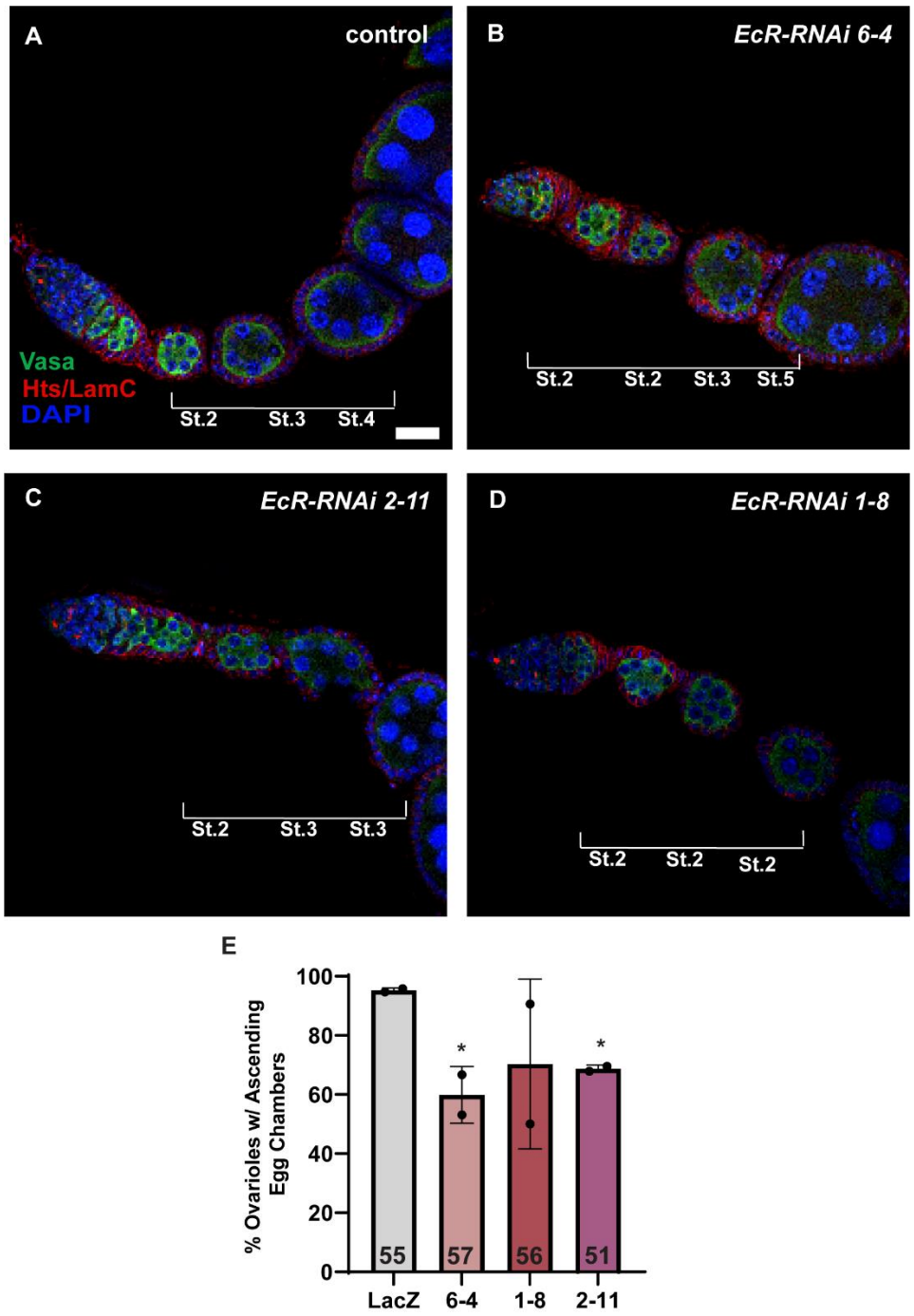


Figure 5: *Driving loss of EcR in 16-Cell Cysts results in growth issues*

[A-D] Ovarioles dissected 5 days after eclosion stained with Vasa (green), anti-Hts (red), anti-LamC (red), DAPI (blue). *OtuGal4::VP16>UASp-lacZ control* [A], *OtuGal4::VP16>UASp-EcR RNAi 6-4* [B], *OtuGal4::VP16>UASp-EcR RNAi 2-11* [C], *OtuGal4::VP16>UASp-EcR RNAi 1-8* [D]. [E] The percentage of egg chambers that properly ascending in numerical order. Error bars show SEM. Students two tailed T-test, *P<0.01, scale bar 10 µm

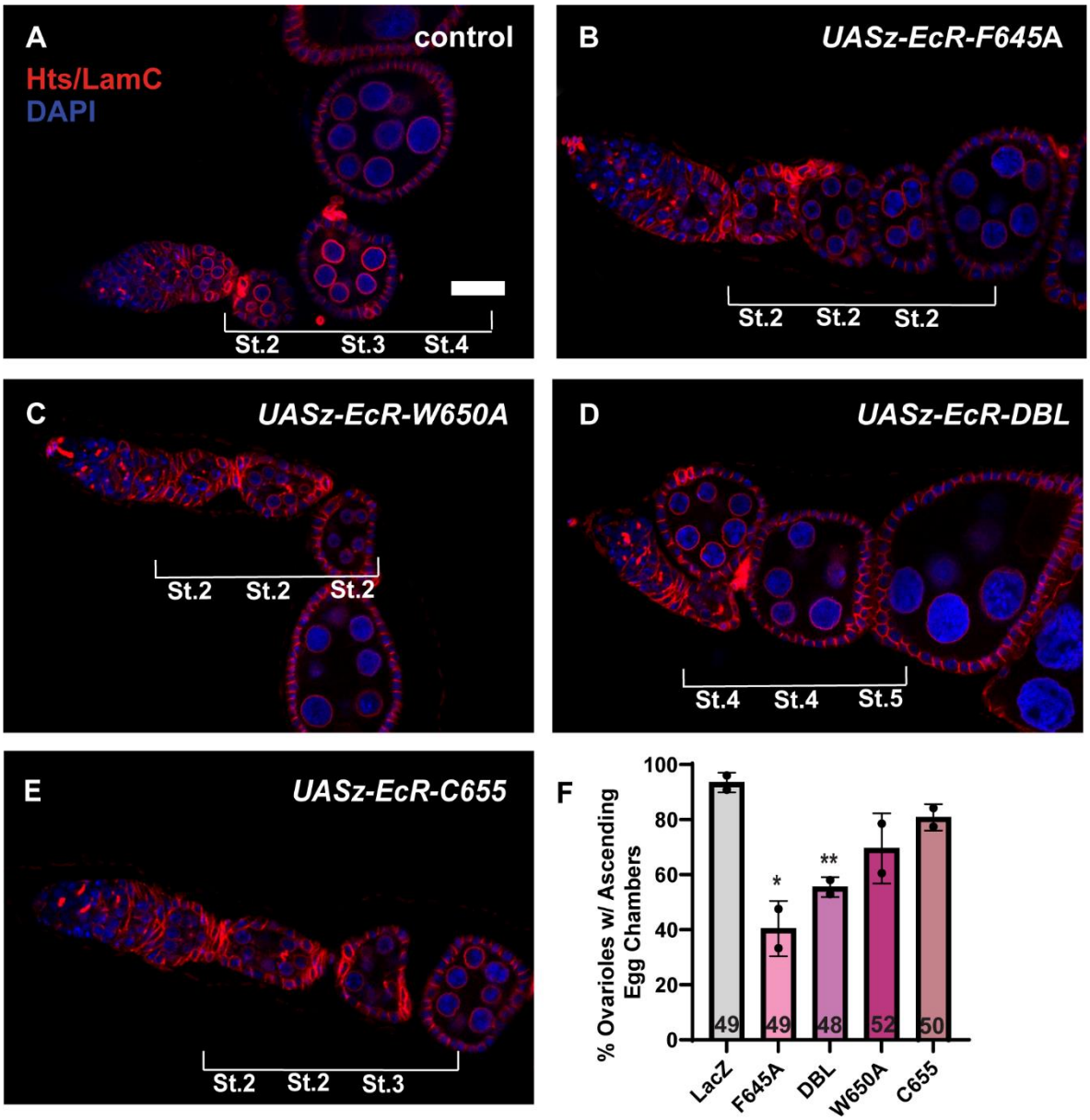


Figure 6: *Loss of EcR through dominate negatives causes growth issues*

[A-E] Ovarioles from females dissected 5 days after eclosion stained with anti-Hts (red), anti-LamC (red), and DAPI (blue). *OtuGal4::VP16>UASp-lacZ control* [A], *OtuGal4::VP16>UASz-EcR F645A* [B], *OtuGal4::VP16>UASz-EcR W650A* [C], *OtuGal4::VP16> UASz-EcR DBL* [D], *OtuGal4::VP16> UASz-EcR-C655* [E]. [F] The percentage of egg chambers that properly ascending in numerical order. Error bars show SEM. Students two tailed T-test, *P<0.01, **P<0.001, scale bar 10 µm.

Knockdown of EcR causes cyst collision defects

The proper encapsulation of 16-cell cysts is a necessary and important process to create a healthy oocyte. As a cyst prepares to enter region 2B, a variety of follicle cell processes occur such as precursor cell division and differential adhesion in which follicular tissues alter their adhesion with neighboring cells (Wu et al., 2008). The 16 cells become separated from the other cysts and start to take form of a disc shape, ingressed by centripetally migrating follicle cells (Chanet & Huynh, 2020). The cyst is then pushed into region 3 of the germarium where it takes a round shape and is encased in a complete monolayer of follicle cells to form an egg chamber.

When knocking down EcR in germ cells, I noticed an intriguing phenotype where the process of encapsulation was disrupted. I crossed all three RNAi lines with *nanosGal4::VP16-GFP* and dissected 5 days after eclosion. I stained ovaries with GFP to visualize all germ cells, as well as 1B1/LamC and DAPI. I then quantified germarium based on if I saw a follicle cell ingression between cysts. Control germarium experienced 17% cyst collisions, likely due to the kinetic process I captured (Figure 7A,E). I found that in all three *EcR-RNAi* lines there was a significant percentage of cyst collisions, appearing most frequently in *EcR-RNAi 6-4* at 45% (Figure 7B-E).

Interestingly, despite the increased number of collision events in the absence of EcR, I did not see a difference in the number of germ cells per egg chamber (control, n=55 ovarioles *EcR-RNAi 6-4*, n=57 ovarioles, *EcR-RNAi 1-8*, n=56 ovarioles, *EcR-RNAi 2-11*, n=51). This suggests that loss of EcR in the germline slows follicle cell centripetal migration rather than blocking it completely.

I then wanted to see if this phenotype occurred when driving knockdown in 16-cell cysts where encapsulation begins. Using *OtuGal4::VP16*, I drove knockdown of *EcR-RNAi 6-4*, *1-8*, and *2-11*. I followed the same experimental plan as when using *nanosGal4::VP16* but stained with the germ cell marker Vasa, 1B1/LamC, and DAPI. Similarly to driving in all germ cells throughout development, *OtuGal4::VP16* caused the same cyst collision phenotype, appearing as significant in all three RNAi lines when compared to the control (Figure 8A-E). To begin characterizing these defects, I used two measurements to quantify the cyst shape and the distance between region 2B and 3 cysts. To quantify cyst shape, I took the aspect ratio of region 3 cysts on the X/Y axis. As expected, control cysts had an aspect ratio close to 1, indicating the cyst is round in shape (Figure 8F). However, when measuring the aspect ratio of *EcR-RNAi* lines, I noticed a decrease in ratio, suggesting that cysts are more lens shaped with an increased Y measurement. *EcR-RNAi 6-4* had an average aspect ratio around 0.8 (Figure 8F). I then wanted to see if there was a difference in the distance of follicle cells separating region 2B and 3 cysts, as this division is normally quite substantial (Chanet & Huynh, 2020). I marked germaria with cyst collisions as a 0uM distance and measured the partition of follicle cells. I found that compared to controls, there was not a huge difference in the distance. Control germaria had an average separation distance of 5uM while RNAi lines had a distance around 3-4uM (Figure 8E). This suggests that the follicle cells could be thinner/flatter or that there are fewer centripetally migrating follicle cells.

I repeated the above experiments using dominate negative and overexpression versions of EcR. The overexpression of EcR using C655 resulted in a similar appearance as controls with 18% of germaria having the cyst collision phenotype. All three dominate negative lines

experienced cyst collisions with the DBL and F645A line appearing most significant (Figure 9A-F). This is consistent with the cyst progression data where these two lines had the strongest phenotype. Shockingly, when performing aspect ratio quantifications on these lines, I did not see a significant difference in cyst shape (Figure 9G). Similarly, the region 2B-3 distance was not notably different when compared to the control distance of 4.8 uM (Figure 9H). Overall, these data sets suggest that EcR is required in germ cells for proper cyst encapsulation.

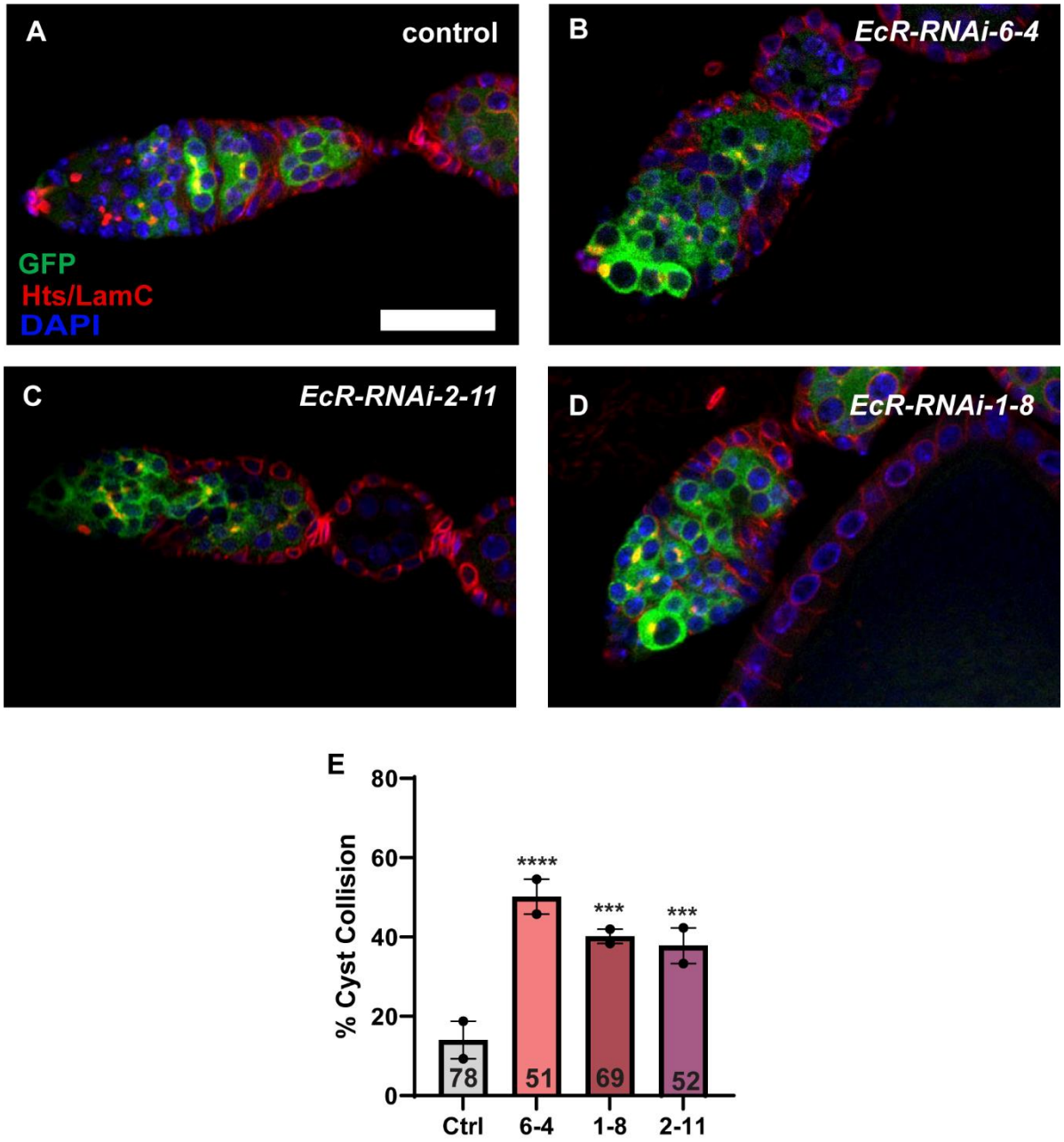


Figure 7: *Loss of EcR in the germline results in delayed cyst encapsulation*

[A-D] Ovarioles dissected 5 days after eclosion stained with GFP (green), anti-Hts (red), anti-LamC (red), DAPI (blue). *nanosGal4::VP16-GFP>UASp-lacZ control* [A], *nanosGal4::VP16-GFP>UASp-EcR RNAi 6-4* [B], *nanosGal4::VP16-GFP>UASp-EcR RNAi 2-11* [C], *nanosGal4::VP16-GFP>UASp-EcR RNAi 1-8* [D]. [E] The percentage of cyst collisions in the germarium. Error bars show SEM. Students two tailed T-test, **P<0.001, ***P<0.0001, ****P<0.00001, scale bar 10 µm.

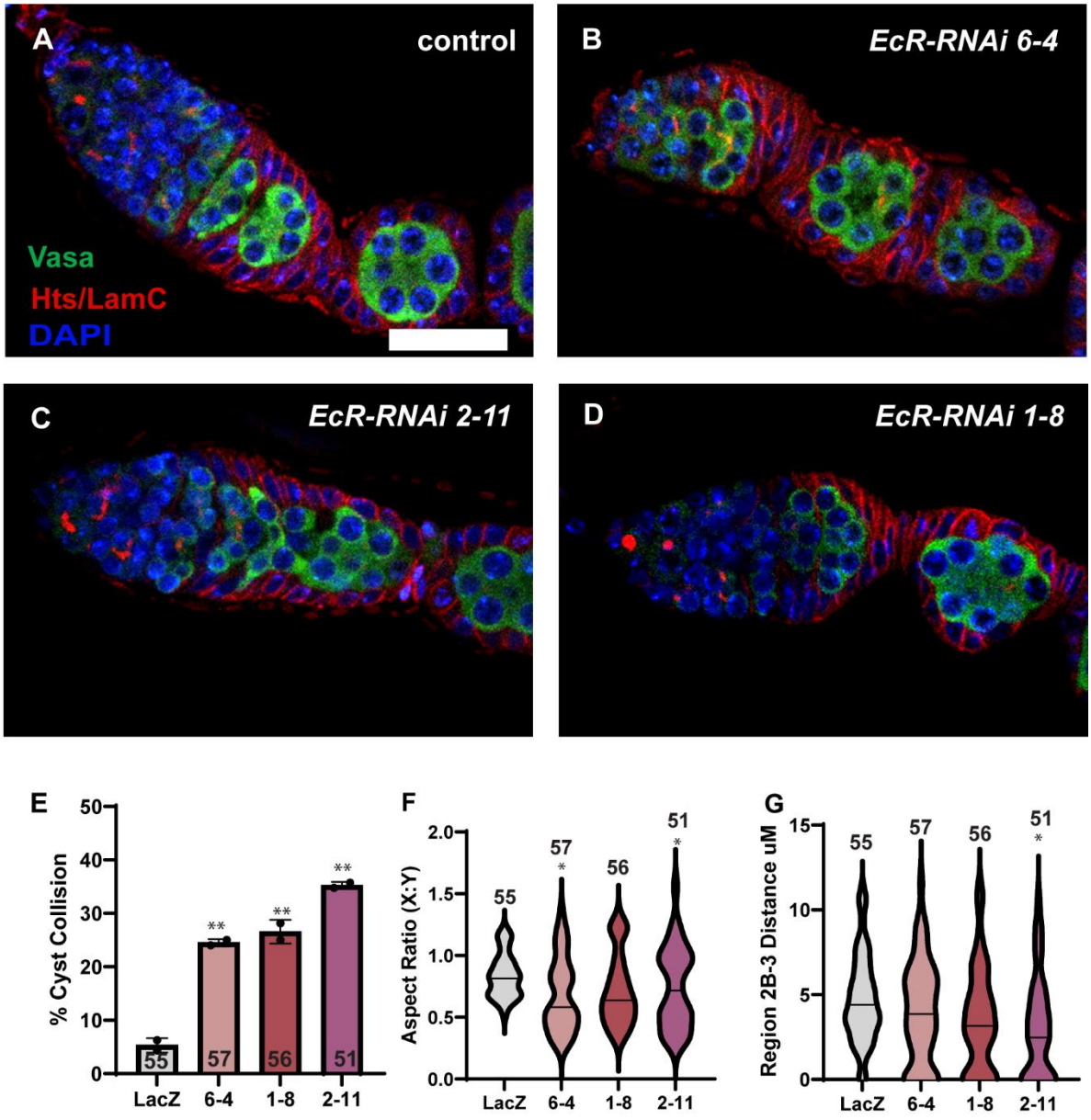


Figure 8: *Loss of EcR in 16-cell cysts results in delayed cyst encapsulation*

[A-D] Ovarioles dissected 5 days after eclosion stained with Vasa (green), anti-Hts (red), anti-LamC (red), DAPI (blue). *OtuGal4::VP16>UASp-lacZ control* [A], *OtuGal4::VP16>UASp-EcR RNAi 6-4* [B], *OtuGal4::VP16>UASp-EcR RNAi 2-11* [C], *OtuGal4::VP16>UASp-EcR RNAi 1-8* [D]. [E] The percentage cyst collisions in germarium. [F] Graph showing various aspect ratio measures (X:Y) for each RNAi line. [G] Graph showing region 2B-3 distances measured in µm. Error bars show SEM. Students two tailed T-test, *P<0.01, **P<0.001, scale bar 10 µm

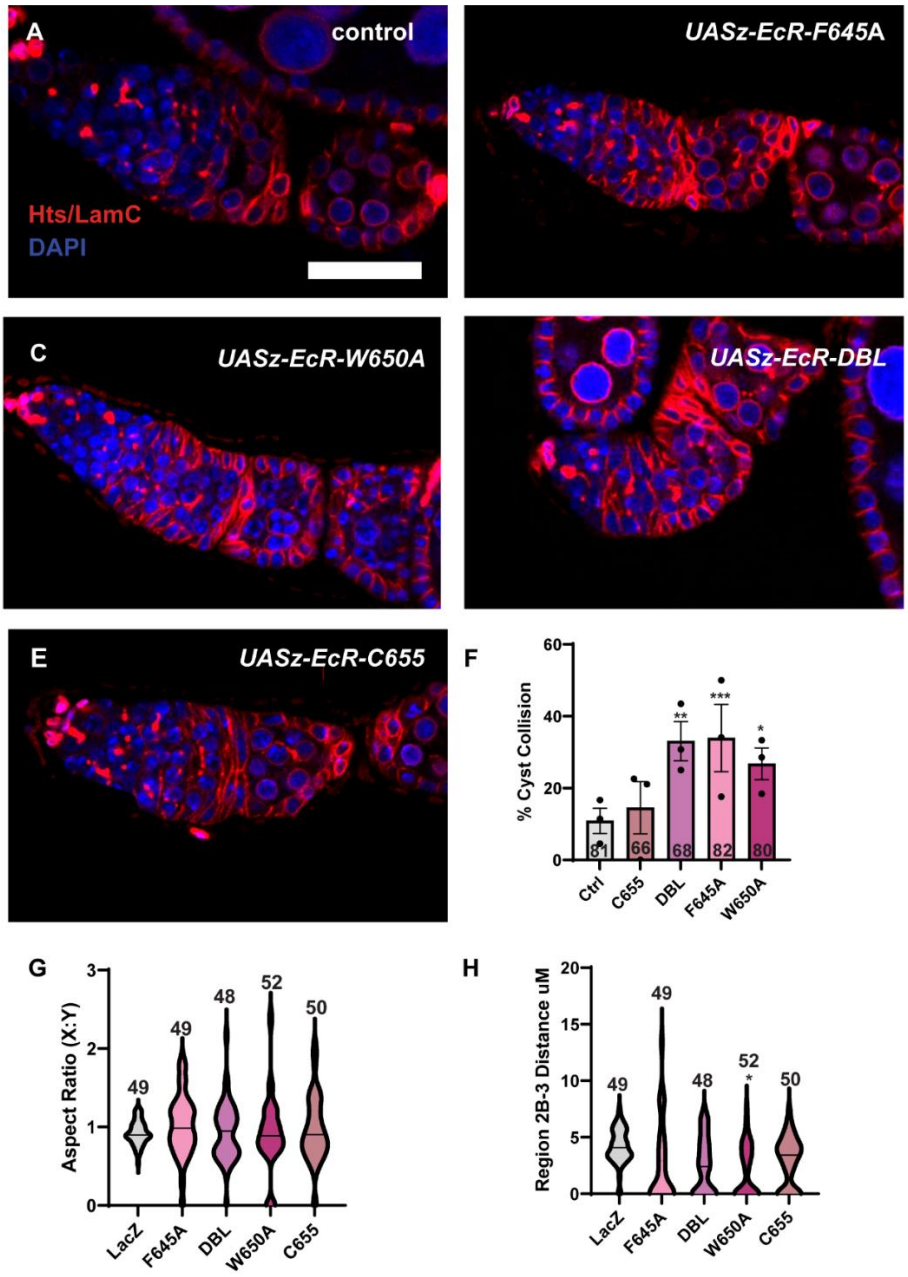


Figure 9: *Loss of EcR using dominate negatives still results in cyst collision issues*

[A-E] Ovarioles from females dissected 5 days after eclosion stained with anti-Hts (red), anti-LamC (red), and DAPI (blue). *OtuGal4::VP16>UASp-lacZ control* [A], *OtuGal4::VP16>UASz-EcR F645A* [B], *OtuGal4::VP16>UASz-EcR W650A* [C], *OtuGal4::VP16> UASz-EcR DBL* [D], *OtuGal4::VP16> UASz-EcR-C655* [E]. [F] The percentage cyst collisions in germarium. [G] Graph showing various aspect ratio measures (X:Y) for dominate negative and overexpression lines. [H] Graph showing region 2B-3 distances measured in µm. Error bars show SEM. Students two tailed T-test, *P<0.01, **P<0.001, ***P<0.0001, scale bar 10 µm.

Loss of EcR in the germline affects follicle cell proliferation

To begin assessing the molecular mechanisms by which EcR controls follicle assembly, I first hypothesized that EcR in germ cells promotes follicle cell proliferation. To test this, I crossed *EcR-RNAi 6-4, UASz-EcR-F645A, and UASz-EcR-DBL* with *nanosGal4::VP16* as those knockdown lines had the most significant phenotypes. I co-labeled EcR with the S-phase marker EdU and the M-phase marker phosopho-HistoneH3 (pHH3) to mark actively proliferating cells. I also stained with Hts/LamC to outline individual follicle cells and DAPI. I quantified the percentage of EdU positive cells by counting total follicle cells from region 2B to 3 and noting how many were EdU positive. pHH3 positive follicle cells were counted but not quantified due to such a small sample of cells going through M-phase even in controls. I found that there was a significant decrease in proliferating follicle cells in all three EcR knockdown lines compared to the control in which 35% were actively proliferating (Figure 10A-E). This suggests that EcR is necessary to stimulate proliferation of overlying follicle cells.

I repeated this experiment with the 16-cell cyst driver, *OtuGal4::VP16* to answer if follicle cell proliferation was still affected when driving in later staged egg chambers. I dissected, stained, and quantified using the same methods as *nanosGal4::VP16.* Interestingly, all three knockdown lines showed similar phenotypes as when using *nanosGal4::VP16*. *EcR-RNAi 6-4* experienced the most significant decrease in follicle cell proliferation with an average around 17%, compared to controls at 36% (Figure 11A-B, E). Overall, this suggests that EcR plays a role in germ to somatic cell communication in order to signal follicle cells to proliferate.

Follicle cell centripetal movement

Recent literature suggests that germ cells actively contract their cytoskeleton to promote their own encapsulation (Chanet & Huynh, 2020). Through pressure release membrane blebs and actomyosin contractile waves, germline cysts can properly sort and pinch off. When altering contractility and membrane blebs in germline cysts, encapsulation defects are generated, identical to those that occur with loss of EcR (Chanet & Huynh, 2020).

Following EcRs role in follicle cell proliferation, I then wanted to begin evaluating if it plays a role in germ cell contractility. First, I used *nanosGal4::VP16-hts.mCherry* to co-express hts while driving EcR knockdown in all germ cells. I stained with Fasciclin 3 to mark follicle cell membranes as well as Vasa to mark germ cells. Unfortunately, this stain set was not rigorous enough to see membrane blebs from the cysts (Supplemental 1A-B). I moved on to another method using the recombination line, *Fas3GFP-nanos-sqh-tdTomato* which endogenously expresses GFP in follicle cells while also expressing *squash* tagged with tdTomato in all germ cells (B. Lin et al., 2022). I plan to use thing line for live imaging of the encapsulation process to visualize the follicle cell invagination around germ cells since each will be distinctly marked (Supplemental 1C-D).

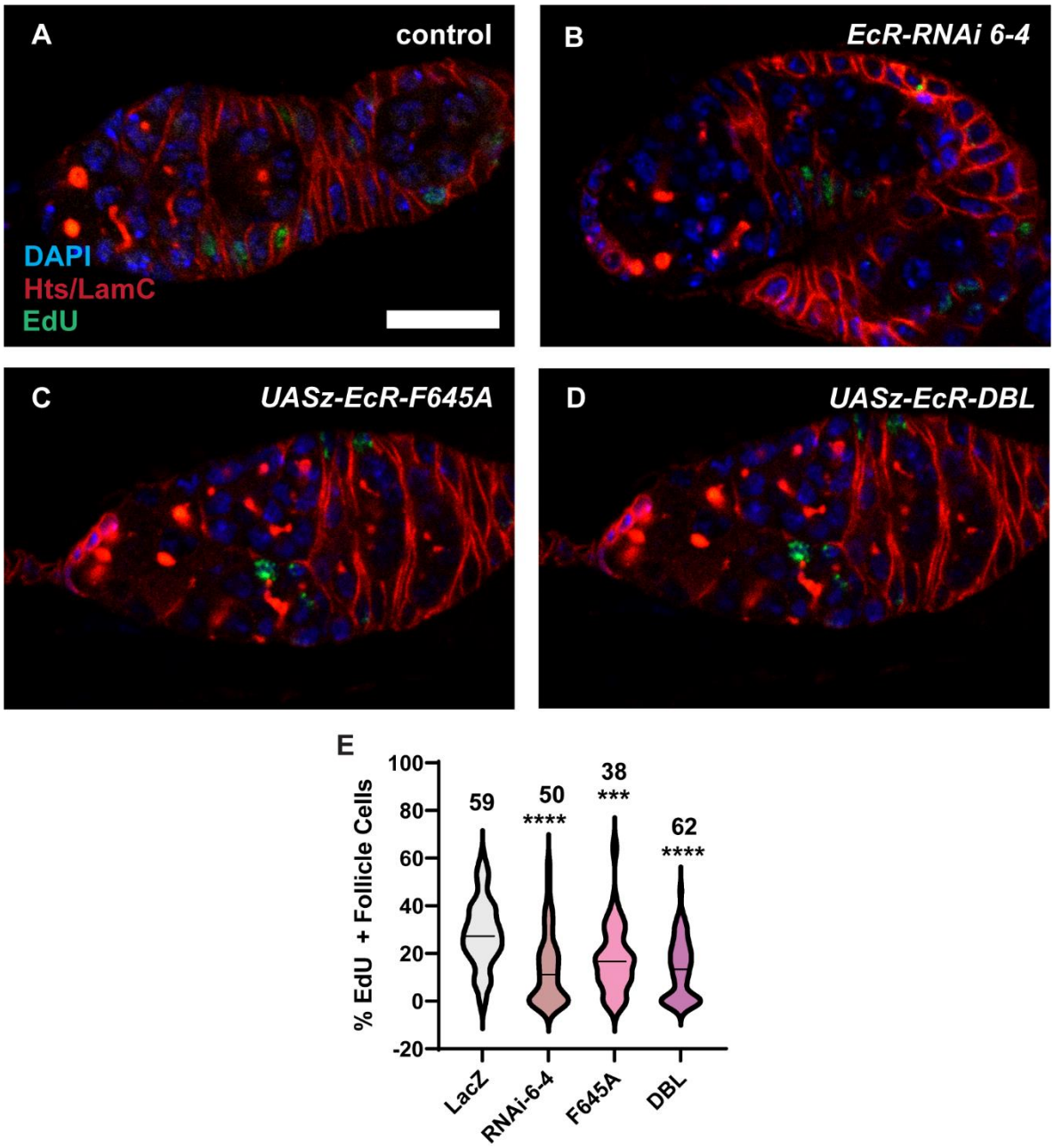


Figure 10: *Loss of EcR in the germline causes decreased follicle cell proliferation*

[A-D] Ovarioles dissected 5 days after eclosion stained with EdU (green), anti-Hts (red), anti-LamC (red), DAPI (blue). *nanosGal4::VP16>UASp-lacZ control* [A], *nanosGal4::VP16>UASp-EcR-RNAi 6-4* [B], *nanosGal4::VP16>UASz-EcR F645A* [C], *nanosGal4::VP16> UASz-EcR DBL* [D]. [E] The percentage EdU positive follicle cells in region2B-3. Students two tailed T-test, ***P<0.0001, ****P<0.00001, scale bar 10 µm.

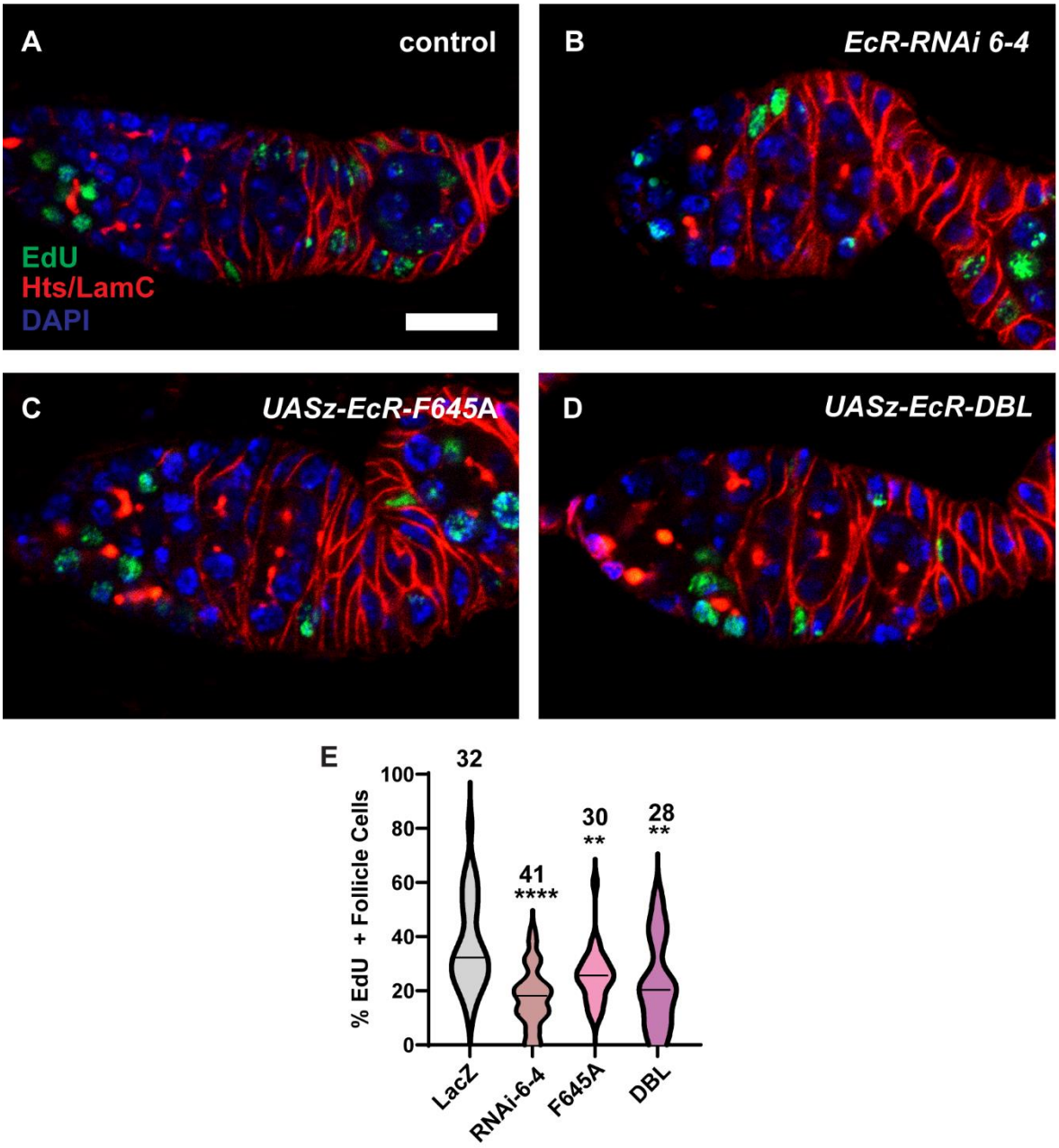


Figure 11: *Loss of EcR in 16-cell cysts causes decreased follicle cell proliferation*

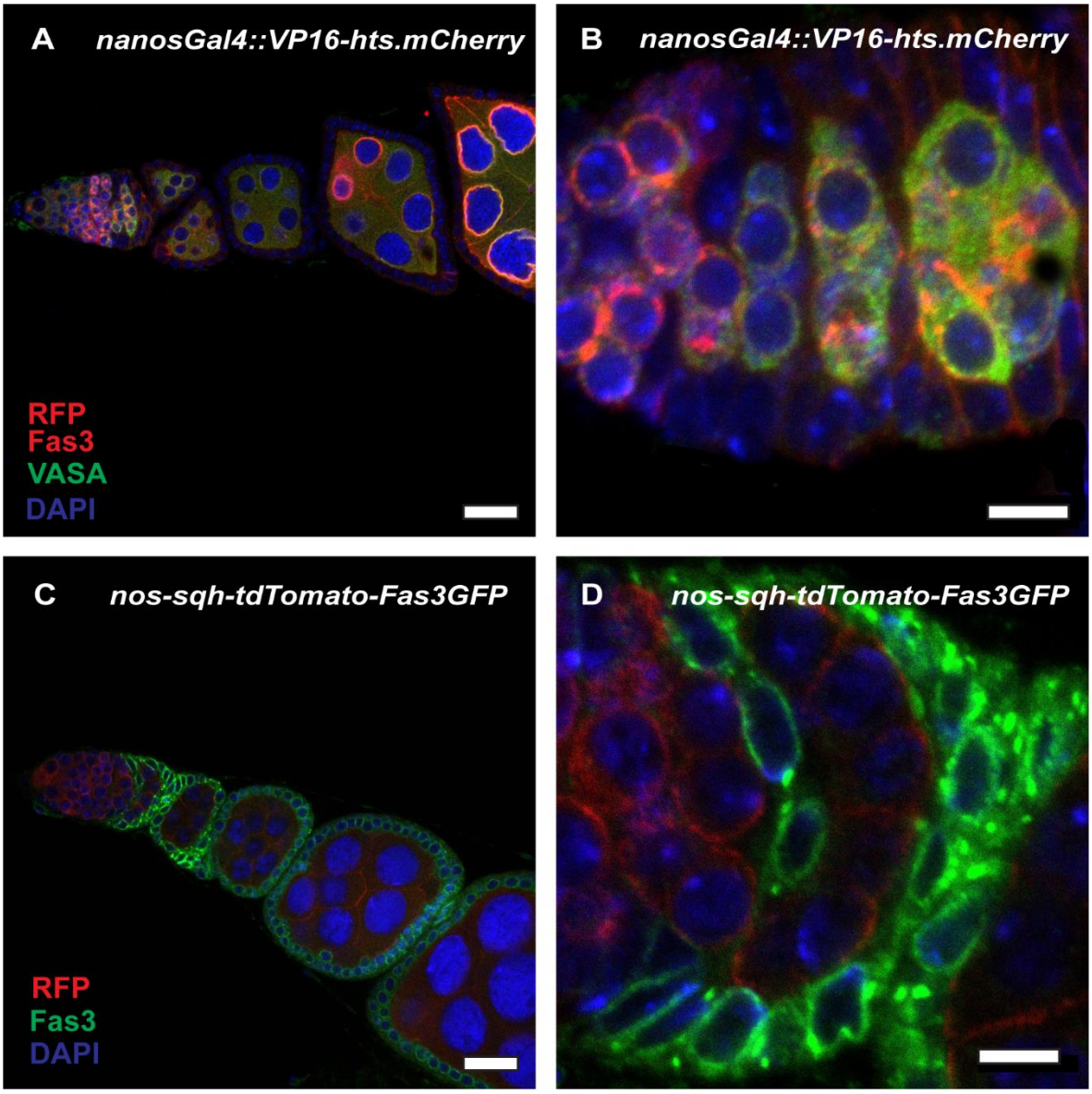
Scale bar, 10 µm [A-D] Ovarioles dissected 5 days after eclosion stained with EdU (green), anti-Hts (red), anti-LamC (red), DAPI (blue). *OtuGal4::VP16>UASp-lacZ control* [A], *OtuGal4::VP16>UASp-EcR-RNAi 6-4* [B], *OtuGal4::VP16>UASz-EcR F645A* [C], *OtuGal4::VP16> UASz-EcR DBL* [D]. [E] The percentage EdU positive follicle cells in region 2B-3. Students two tailed T-test, **P<0.001, ****P<0.00001, scale bar 10 µm.

DISCUSSION

My study reveals a novel role of *Ecdysone receptor* in regulating germ cell encapsulation by somatic follicle cells. Additionally, it suggests EcR plays a role in mediating germ to somatic cell communication in order to signal follicle cells to proliferate. Although Ecdysone has never been implicated in these processes, literature suggests possible mechanisms for its role in intracellular signaling. To properly encapsulate a healthy oocyte, a variety of signaling pathways are needed. One of the most notable signals is the Notch/Delta ligand. Notch signaling, activated by a germline Delta ligand signal, has been shown to influence various somatic cell fates (Assa-Kunik et al., 2007). It has also been implicated in follicle cell patterning when Notch is opposed by JAK/STAT signals. Another signal involved in germ to somatic cell communication is the ligand stet, received by epidermal growth factor receptor (Egfr) on somatic cells. Stet has been implicated in germ cell differentiation as well as germ cell encapsulation (Schulz et al., 2002). It is possible that EcR generates an intracellular ligand such as Delta or stet to stimulate proliferation of follicle cells. It cannot be said for certain whether EcR directly influences follicle cell proliferation. However, when the stalling of cyst growth is considered, it suggests loss of EcR generates a lack of follicle cells to properly and timely encapsulate cysts. To verify this model in future experiments, the X-gal system will be useful to quantify the timing of cysts as they exit the germarium (Schulz et al., 2002). Additionally, it would be interesting to perform egg counts across all EcR knockdown lines to answer if timing is affected.

Perhaps another model is the role of EcR in germ cell cortical contractility. The morphogenetic characteristic of follicle cells is extremely important for proper encapsulation of germline cysts (Horne-Badovinac & Bilder, 2005). Although knockdown of EcR does not result in abnormal numbers of packaged cysts, the lack of invaginating follicle cells makes for an intriguing phenotype. Although numerous mutations have been identified in cyst collision phenotypes, the mechanisms by which follicle cells recognize and individualize a cyst are widely unknown. Recent literature suggests that through membrane blebs and actomyosin contractile waves, germline cysts are properly sorted (Chanet & Huynh, 2020). Since the experiments performed in this paper were not rigorous enough to visualize this process, future experiments using the developed recombinant lines will be necessary to answer this question. By using live imaging techniques, we will be able to visualize this dynamic process to see if contractility is decreased. Additionally, various experiments using known influencers of germ cell cortical contractions such as *chic* and *zipper* can be performed with EcR knockdown to observe if cyst collision phenotypes are increased.

Here, I describe two potential mechanisms for the defects seen in egg chamber encapsulation when depleting EcR in the germline. It is shown that loss of EcR results in slowed growth phenotypes accompanied with cyst collisions and a decrease in follicle cell proliferation. This demonstrates that EcR is necessary in germ cells to mediate an indispensable follicle cell process.



Supplemental 1: *Testing germ and somatic cell markers for live imaging*
[A-B] *nanosGal4::VP16-htsmCherry>UASp-LacZ* stained with RFP (red), Fas3 (red), Vasa (green), DAPI (blue). [C-D] *nanos-squashTd-Tomato-Fas3GFP* stained with RFP (red), Fas3 (green), DAPI (blue). Scale bar, 10 µm

Materials and Methods

Drosophila Strains and Husbandry

Drosophila stocks were raised and maintained at 22-25°C in a medium consisting of cornmeal, molasses, yeast, and agar. Crosses were tossed into new medium every 3 days. On the day of eclosion, flies were sorted and fed fresh wet yeast paste for 3-6 days up until their dissection. All stocks and reagents can be found in Table 1.

Table 1

Fly Stocks and Other Reagents

Reagent Type	Designation	Source	Identifiers	Additional Information
Genetic reagent (*Drosophila melanogaster*)	UASp-LacZ	D. Drummond-Barbosa	N/A	
Genetic reagent (*Drosophila melanogaster*)	UASz-EcR.B1(deltaC655)/CyO_1	Bestgene (Jung et al, in prep)	N/A	EcR.B1
Genetic reagent (*Drosophila melanogaster*)	UASz-EcR.B1(F645A)/CyO_1	Bestgene (Jung et al, in prep)	N/A	EcR.B1
Genetic reagent (*Drosophila melanogaster*)	UASz-EcR.B1(W650A)/CyO_1	Bestgene (Jung et al, in prep)	N/A	EcR.B1
Genetic reagent (*Drosophila melanogaster*)	UASz-EcR.B1(DBL)/CyO_1	Bestgene (Jung et al, in prep)	N/A	EcR.B1
Genetic reagent (*Drosophila melanogaster*)	Nanos-Gal4::VP16	D. Drummond-Barbosa	N/A	(Rørth, 1998; Van Doren et al., 1998)
Genetic reagent (*Drosophila melanogaster*)	P{otu-GAL4::VP16.R}1, w1118	Bloomington	#58424	
Genetic reagent	UAS-EcR-1-8 (A)	Bestgene (Phipps et al, in prep)	N/A	P-Val22

(*Drosophila melanogaster*)				
Genetic reagent (*Drosophila melanogaster*)	UAS-EcR-2-11(A)	Bestgene (Phipps et al, in prep)	N/A	P-Val22
Genetic reagent (*Drosophila melanogaster*)	UAS-EcR-6-4 (A)	Bestgene (Phipps et al, in prep)	N/A	P-Val22
Genetic reagent (*Drosophila melanogaster*)	NanosGal4::VP16-hts.mCherry	Anna Williams		Produced by Ables Lab
Genetic reagent (*Drosophila melanogaster*)	Fas3-GFP / (CyO) ; Dr / TM6, DfdGFP	Lindsey Plasschaert		Received from Dr. Lauren Anllo
Genetic reagent (*Drosophila melanogaster*)	Nos-myosin-II-tdTomato-P2A-tdKatushka2-CAAX	Benjamin Lin of Ruth Lehmann's Lab		Lin et al., 2022
Genetic reagent (*Drosophila melanogaster*)	Fas3GFP-nanos-sqh-tdTomato	Lindsay Swain		Produced by Ables Lab
Antibody	Chicken anti-GFP	Abcam	#AB13970	1:2000
Antibody	Mouse anti-Hts	DSHB	RRID: AB_528070	1B1; 1:10
Antibody	Mouse anti-LaminC	DSHB	RRID: AB_528339	LC28.26; 1:100
Antibody	Rabbit anti-DCP-1	Sigma	#AV39353	1:100
Antibody	Rat anti-Vasa	DSHB	RRID: AB_760351	1:50
Antibody	AlexaFluor 488	Life Technologies		Species Specific
Antibody	AlexaFluor 568	Life Technologies		Species Specific
Dye	4'-6-diamidino-2-phenylindole (DAPI)	Sigma		0.5 μg/ml in 0.1% PBS-Triton X-100
Commercial Kit	Click-iT EdU Cell Proliferation Kit for Imaging, Alexa Fluor 647 dye	Fischer Scientific	Invitrogen C10340	

Other	Grace's medium	Caisson Labs		
Other	Phosphate-buffered saline (PBS)	Fisher Scientific		pH 7.4
Other	Blocking Solution	Sigma, MP Biomedicals		5% bovine serum albumin (BSA), 5% normal goat serum (NGS), 0.1% PBS-Triton X-100
Other	Grieder Blocking Solution	MP Biomedicals		5% normal goat serum, 0.1% PBS-Triton X-100
Other	Mounting Media	Sigma		90% glycerol; 20% n-propyl gallate

Immunofluorescence and Microscopy

Normal Protocol

Ovaries were dissected and teased apart in chilled Grace's medium and gently placed into 3% BSA pre-coated tubes. Samples were then fixed in cold 5.3% formaldehyde in Grace's medium for 13 minutes on a rotating nutator at room temperature. Samples were immediately rinsed and washed three times for 20 minutes with phosphate-buffered saline (PBS) with 0.1% Triton X-100 on a rotating nutator. They were then blocked for three hours in blocking solution [5% bovine serum albumin (BSA), 5% normal goat serum (NGS), 0.1% PBS-Triton X-100] at room temperature. Primary antibodies were diluted in blocking solution and incubated overnight at 4°C on a rotating nutator. All primary antibodies were removed and followed by four, 20-minute washes with 0.1% PBS-T. Secondary antibodies were diluted in blocking solution and placed on a nutator at 4°C overnight in a dark environment. All secondary antibodies were removed, and samples were washed four times for 20 minutes with 0.1% PBS-T. Ovary samples were stained with 0.5 µg/ml 4'-6-diamidino-2-phenylindole (DAPI) in 0.1% PBS-T. Samples were stored in mounting media (90% glycerol and 20% n-propyl gallate) at 4°C and mounted on a glass slide for imaging. Confocal z-stacks of 1 µm were taken on the Zeiss LSM 700 laser

scanning microscope using ZEN Blue software. Images were analyzed on Zeiss ZEN Blue software and enhanced using the Adobe Photoshop Creative Suite.

Grieder Protocol

All above steps were followed with exception to both room temperature dissections and Grace's medium. Additionally, Grieder blocking solution [5% normal goat serum (NGS) and 0.1% PBS-Triton X-100] was used to block and dilute primary and secondary antibodies.

Analysis and Statistics

To quantify ascending egg chambers, we took two measurements to stage cysts (Spradling, 1993). These measurements were taken using the "Line" tool in ZEN Blue (Zeiss) imaging software and recorded in nanometers. Both an X and Y measurement were taken at the most midway Z stack plane. If cysts were in between two stages, the closer of the two stages were recorded. Cysts were considered "Ascending" if egg chambers proceeded in a linear set (2, 3, 4, etc.)

Cyst cell numbers were counted using the "Click Counter" tool in ZEN Blue (Zeiss) at each plane of the Z stack image to ensure all cells were included.

Cyst collision defects were quantified by determining if there was a present follicle ingression in Region 2B/3 to separate 16-cell cysts. If possible, more strenuous quantification was performed to determine both aspect ratio and separation distance of the cysts. At the midpoint of the germarium, region 3 cysts were measured on an X/Y axis from the interior of the germ cell-somatic cell border. X:Y ratios were analyzed for each spherical cyst. The separation distance of cysts was measured from Region 2B to Region 3 by using the same "Line" tool. If the two cysts had no partitioning follicle cells in between them, the distance was recorded as 0.

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