Identification of a novel autonomous role for Ecdysone Receptor during *Drosophila* ovarian germ cell differentiation.

By
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ABSTRACT

A tightly regulated network of signals coordinates the division of *Drosophila* germline stem cells (GSCs) and differentiation of their daughter cells to produce viable oocytes. The steroid hormone, ecdysone, is known to promote GSC self-renewal; however, due to lack of compatible genetic tools, potential autonomous roles in the germline have not been fully evaluated. Ecdysone elicits a diverse array of transcriptional responses by binding to a heterodimeric complex composed of Ecdysone Receptor (EcR) and Ultraspiracle (Usp). To elucidate whether EcR facilitates autonomous reception of ecdysone in the germline, we built germline-compatible genetic tools to manipulate EcR levels or activity. Depleting *EcR* mRNA or loss of EcR ligand binding in the germline caused a loss of GSCs over time, demonstrating that it is necessary for GSC self-renewal. Further, over-expressing either EcR.A or EcR.B1 isoforms resulted in undifferentiated germ cell tumors and decreased numbers of GSCs. This is a ligand-dependent function of EcR, as over-expression of EcR that cannot bind ecdysone suppressed tumor development. By restricting over-expression to later stages of the germline, we found that EcR is sufficient to induce tumors only when over-expressed in the GSCs and early daughter cells. In tumorous ovaries, stem-like cells were also identified outside of their normal stem cell niche, suggesting that these cells remain competent to respond to BMP signals. Using single-cell RNA-sequencing to compare EcR over-expressing and Tkv over-expressing ovaries, we found remarkably similar transcriptomes in heterogenous populations of germ cells. These data suggest

that EcR promotes differentiation of germ cells by directly regulating components of the BMP signaling pathway and that low levels of EcR expressed in wildtype GSCs are sufficient for ligand-dependent activation of an ecdysone-responsive transcriptional program. Altogether these data reveal a novel, autonomous role of EcR in GSC maintenance and regulation of differentiating daughter cells.

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LIST OF ABBREVIATIONS

GSC	Germline stem cell
EcR	Ecdysone Receptor
usp	ultraspiracle
AF1	Activation function 1
Ftz-f1	Fushi tarazu transcription factor 1
DBD	DNA binding domain
LBD	Ligand binding domain
BMP	Bone morphogenetic protein
UAS	Upstream activating sequence
CyO	Curly of Oster
OE	Over-expression
DN	Dominant negative
Hts	Hu li tai shao
LamC	LaminC
LamB	LaminB
nos	nanos
dpp	decapentapalegic
gbb	Glass bottom boat
Tkv	Thick veins
Sax	saxophone
Mad	Mothers against decapentaplegic
pMad	Phosphorylated Mad

Bam	Bag of marbles
Dad	Daughters against dpp
PHH3	Phospho-histone 3
bgcn	Benign gonial cell neoplasm
Mei-p26	Meiotic protein 26
Fs(1)k10	Female sterile (1) k10
side	sidestep
ple	pale
esg	escargot
scrt	scratch
Phm	phantom
sad	shadow
shd	Shade
Nvd	Neverland
Dib	Disembodied
Spo	Spooky
dae	Days after eclosion

Introduction

The steroid hormone ecdysone regulates diverse developmental processes across the lifespan, including tissue remodeling, tissue growth, developmental timing, and female fertility (Ables & Drummond-Barbosa, 2017). Ecdysone titers act as a temporal cue to initiate progression through larval stages, pupariation, and the final metamorphic transition to adulthood. Both the timing and level of ecdysone are critical for stage-appropriate events (Morrow & Mirth, 2024). Ecdysone levels are, in part, regulated by its synthesis. Dietary cholesterol is modified by a series of enzymes encoded by the Halloween gene family to produce ecdysone (Rewitz et al., 2006). In larval and pupal stages, the prothoracic gland is the primary source of ecdysone synthesis, where it is released into the hemolymph and circulated throughout the body. In the adult female, the ovary is the largest source of ecdysone production and loss of ecdysone biosynthesis prohibits progression of oogenesis (Swevers, 2018).

Developmental responses to ecdysone are initiated through an elaborate transcriptional response mediated by the Ecdysone Receptor (EcR). As a heterodimer composed of two nuclear receptors, encoded by the genes *EcR* and *ultraspiracle* (*usp*), the EcR complex regulates distinct temporal and spatial expression of genes through activation and repression (Uyehara and McKay et al., 2019). Context-dependent responses are, in part, due to the existence of three distinct EcR protein isoforms (EcR.A, EcR.B1, and EcR.B2), each with differential expression and transcriptional activity (Schubiger et al., 2003). The A and B1 isoforms share conserved DNA and ligand binding domains but have alternatively spliced N-termini (Talbot et al., 1993). The differing N-termini, also known as activation function 1 (AF1) region, suggests an isoform-specific role; however, to what extent the EcR isoforms regulate biological processes remains largely unknown (Schauer et al., 2011).

EcR-mediated signaling is critical for oogenesis in *Drosophila melanogaster*. Oogenesis begins in adults with a germline stem cell (GSC) population that resides in a specialized niche within the germarium at the anterior tip of each of ovarian subunit, called ovarioles (McLaughlin & Bratu, 2015; Hinnant et al., 2020). GSCs divide asymmetrically to produce a cystoblast, which continues to undergo four mitotic divisions with incomplete cytokinesis to produce a 16-cell cyst (de Cuevas et al., 1997). Within the interconnected cyst, one cell will commit to becoming the oocyte and the remaining 15 will become nurse cells. Follicle cells at the posterior end of the germarium envelop the cyst and bud it off from the germarium to form an egg chamber. In the egg chamber, nurse cells produce mRNA and proteins for the oocyte to uptake then undergo programmed cell death, producing a fully developed oocyte.

Analysis of EcR isoform specificity in the adult ovary has not been accomplished, largely because loss-of-function mutations specifically disrupting *EcR.A*, *EcR.B1*, or *EcR.B2* result in larval lethality (Bender et al., 1997). However, loss-of-function experiments using a temperature-sensitive allele of *EcR* (EcR^{A483T}) permits temporal control over *EcR* knock-down and thus, allows flies to develop to adulthood before loss of gene function (Bender et al., 1997). Utilizing EcR^{A483T}, a global loss-of-function mutant that disrupts the common ligand binding domain, it was established that EcR is critical for egg chamber development and survival (Buszczak et al., 1999; Carney & Bender, 2000) and GSC self-renewal (Ables & Drummond-Barbosa, 2010; Morris & Spradling, 2012; Konig et al., 2011). However, these experiments could not distinguish whether EcR was autonomously required in the germline or the somatic cells for the observed effects on oogenesis.

To begin to address which ovarian cells require EcR, the Montell and Cherbas labs used the UAS/Gal4 system to over-express a dominant-negative EcR transgene in ovarian border cells,

which form the anterior margin of the eggshell and the sperm entry point (Bai et al., 2000; Jang et al., 2000; Cherbas et al., 2003; Bhattacharya & Starz-Gaiano, 2024). These experiments demonstrated that EcR is necessary to provide a timing signal necessary for the proper migration of border cells, and thus, for female fertility. EcR is also necessary in ovarian follicle cells for several other processes including follicle cell migration and shape, oocyte lipid accumulation, and ovulation (Manning et al., 2017; Knapp & Sun, 2017; Romani et al., 2016; Sieber & Spradling, 2015). Using a similar experimental strategy, the Gilboa and Shcherbata labs found that EcR is also critical for primordial germ cell differentiation. Expression of the EcR dominant-negative transgenes in the somatic cells of the larval ovary caused precocious differentiation of primordial germ cells and subsequent abnormalities in GSC niche formation (Konig et al., 2011; Gancz et al., 2011; Yatsenko & Shcherbata, 2018).

A direct role for EcR in germ cells has not yet been established. The role of EcR within the germline has been largely presumed upon identification of a few EcR response genes expressed in the ovary (McDonald et al., 2019; Ables and Drummond-Barbosa, 2017). Canonical early targets E74, E75, and Broad are all expressed in germ cells, suggesting that EcR activates their expression (Buszczak et al., 1999). Moreover, E74 and EcR co-receptor Usp are both necessary in GSCs for their maintenance (Ables and Drummond- Barbosa, 2010). E78 is also essential in germ cells for cyst viability, and non-autonomously in somatic cap cells for stem cell niche establishment, GSC maintenance, and cyst viability (Ables et al., 2015). E74 and E75 are expressed at high levels beginning in 16 cell cysts and E75 null germline clones arrest during mid-oogenesis (Terashima & Bownes, 2006; Buszczak et al., 1999). Other ecdysone responsive nuclear receptors, such as Fushi tarazu transcription factor 1 (Ftz-f1) and HR3, show distinct and controlled expression throughout the ovary, although their role is largely undefined (McDonald

et al., 2019). It has yet to be directly assessed whether these ecdysone responsive genes are regulated by EcR in the germline. Furthermore, although some roles of EcR in the germline can be implied by manipulations to canonical target genes, the direct and autonomous role EcR plays in the germline has not been evaluated. This is largely due to the lack of compatible germline genetic tools, since the core Hsp70 promoter used in the majority of UAS and transgenic constructs is silenced by piRNAs (DeLuca & Spradling, 2018).

Here, we present evidence that EcR is necessary and sufficient in ovarian GSCs for their self-renewal. Using novel genetic tools to over-express EcR, we find that it controls a stem cell transitional state. Utilizing single-cell RNA-sequencing, our data suggests that ecdysone signaling controls germ cell fate through the bone morphogenetic protein (BMP) signaling pathway. These data underscore previously unappreciated roles of steroid hormones in stem cell maintenance and differentiation in adult tissues.

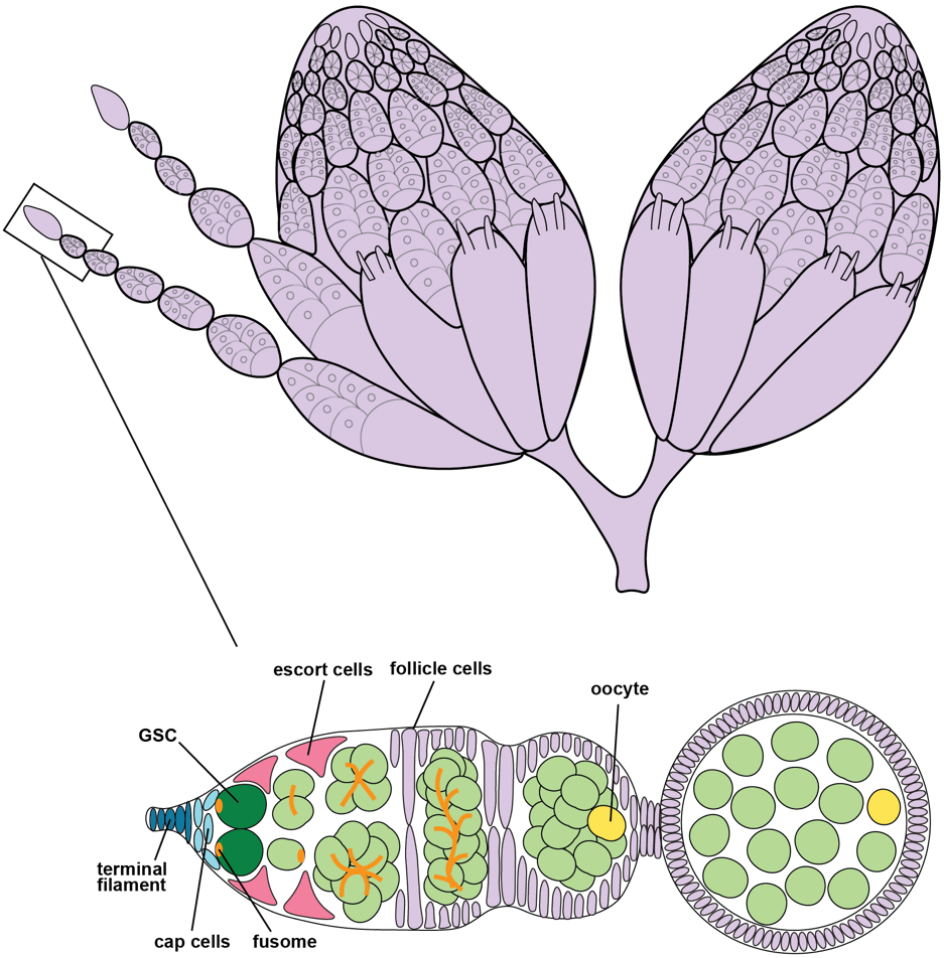

Figure 1: *Drosophila* ovary and germaria diagram. Each ovary is composed of ovarioles, connected chains of developing egg chambers arising from a single germarium. At the anterior tip, germline stem cells (GSCs, dark green) are attached to cap cells (pink) and have a specific organelle (the fusome, orange) that is useful for identification. GSCs divide to form the cystoblast (lime green), which undergoes four rounds of synchronous mitotic divisions. The resulting 16-cell cyst consisting of 15 nurse cells and one oocyte (yellow) becomes enveloped by a layer of follicle cells (light blue) to produce an egg chamber.

RESULTS

Generation of new genetic tools to manipulate EcR in the germline.

To unveil the potential germ cell autonomous role of EcR, we designed and built new genetic tools to manipulate EcR. The Gal4/UAS system allows for spatio-temporal controlled expression of transgenic constructs (Caygill & Brand, 2016). Gal4, a yeast transcription factor, can be expressed under cell or tissue specific enhancers. Gal4 can then bind Upstream Activating Sequence (UAS) elements to turn on transcription of those target genes. Many of the current tools to manipulate EcR under UAS control have a heat shock promoter that allows for expression in somatic cells but becomes suppressed in germ cells (DeLuca & Spradling, 2018). A new transgenic vector, UASz 1.0, has a different promoter and allows for effective expression in the germline.

In the UASz construct, we built an over-expression construct for an EcR protein that can bind ecdysone and is functionally undistinguishable from wildtype protein (*UASz-EcR.B1*$^{\Delta C655}$ = *EcR.B1* and *UASz EcR.A*$^{\Delta C627}$ = *EcR.A*) (Figure 2B). We also created three additional transgenes of EcR dominant negatives that are unable to transactivate transcription due to mutations in their ligand binding domain (Cherbas et al., 2003) (Figure 2A-B). When these dominant negatives are over-expressed, they outcompete endogenous EcR protein for ligand binding.

Although these constructs have previously been described to effectively knockdown ecdysone signaling, we first confirmed that they function in the UASz background. To do this, the EcR.B1 over-expression and dominant negative constructs were expressed under *Actin-Gal4*. Actin expresses in all somatic cells throughout all stages of development. EcR is necessary for the progression through pupariation, so we expected flies expressing the dominant negatives

would not survive into adulthood, while excess functional EcR protein should not inhibit fly development (Cherbas et al., 2003). We found that expressing the UASz dominant negatives under *Actin-Gal4* was in fact lethal. Expression of EcR.B1 however was not, this showing these tools function similar to the original transgenes.

Over-expression of EcR.B1 in germ cells causes tumors.

EcR is expressed at apparently equal levels in GSCs, their dividing daughters, and in surrounding somatic cells of the germarium (Martin et al., 2022; Schwedes et al., 2011). We thus sought to test whether over-expression of EcR in germ cells impacted germline development. To confine expression to germ cells, we used *nanos-Gal4::VP16* (*nos-Gal4)*, a strong driver that is maximally expressed in GSCs and their dividing daughters. Driving *UASp-GFP* allowed us to label germ cells with a fluorescent tag, while immunostaining with anti-Hts and anti-LaminC allowed for visualization of fusomes and nuclear membranes, and DAPI stained nuclei. As expected, expression of LacZ alone yielded ovarioles with 2-3 GSCs per germarium, mitotically dividing cysts, and egg chambers produced from the niche (Figure 3A). In contrast, over-expression of *EcR.B1* in all germ cells under *nanos-Gal4* resulted in germ cell tumors (Figure 3B). Instead of various stages of cyst development (2, 4, 8, and 16-cell) in the germarium, there is no identifiable post-mitotic, 16-cell cysts; rather, only cells with a round, dotted fusome indicative of cystoblasts. We identified egg chambers by larger nurse cell nuclei and approximate correct number of germ cells. As a result of the improper cyst development, egg chamber production was significantly reduced (Figure 3E).

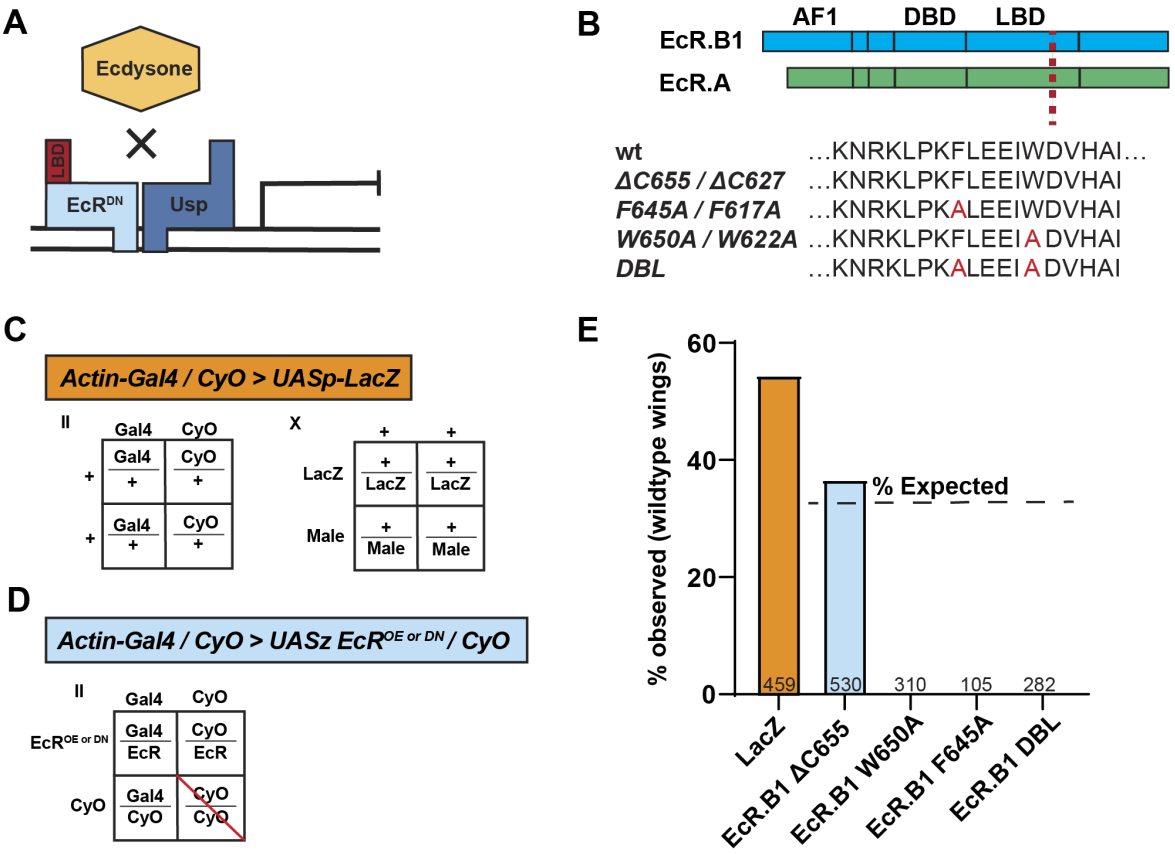

Figure 2: Generation of new genetic tools to manipulate EcR in the germline. (A) Diagram of EcR dominant-negative function, where the the ligand binding domain inhibits ability of ecdysone binding. (B) EcR.B1 and EcR.A share a common DNA binding domain (DBD) and ligand binding domain (LBD) but have different activation function 1 (AF1) regions. Over-expression and dominant negative sequences have an early termination. In red are the amino acid substitutions made. (C-D) Punnett squares representing predicted genotypes of lethality screen progeny. *Curly of Oster* (*CyO*) is a wing trait used to identify flies that do not have the *Gal4* gene. If *EcR* over-expression (OE) or dominant-negative (DN) expression is non-lethal, one third of progeny should have wildtype wings. (E) Bar graph of percent of progeny from each cross that have wildtype wings and thus the *EcR* transgene. Sample size is represented at the base of each bar.

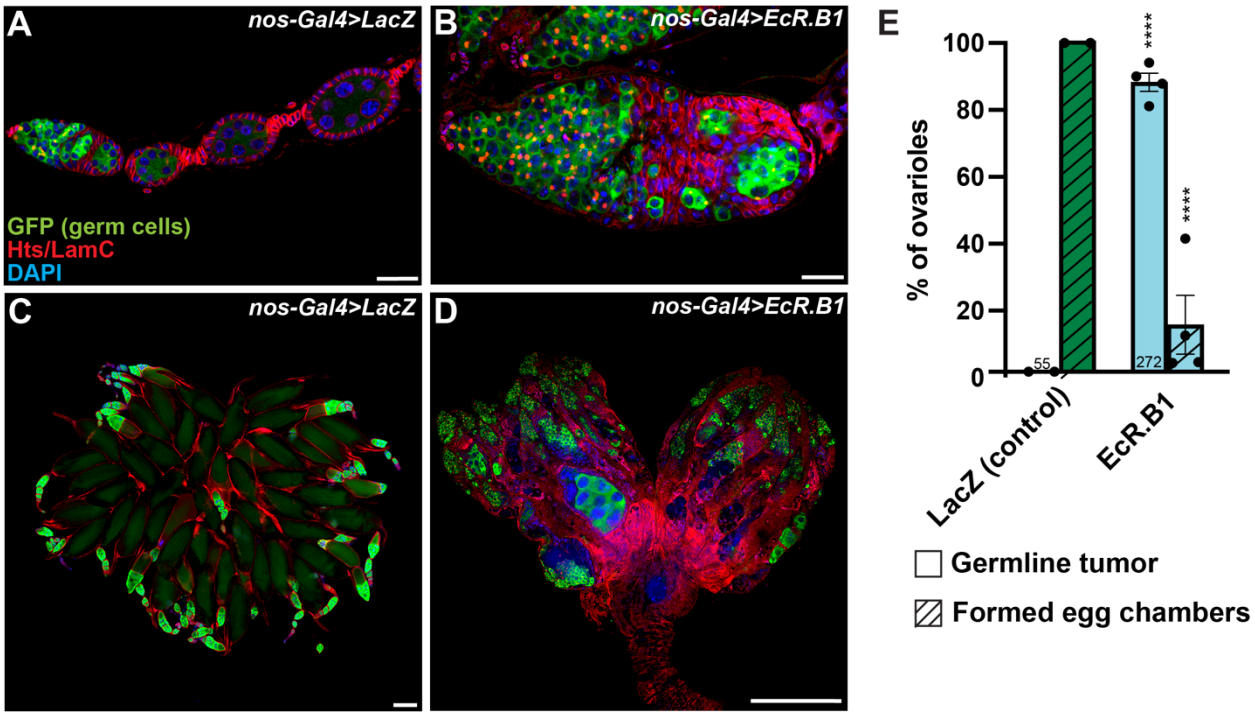

Figure 3: Over-expression of *EcR.B1* in germ cells causes tumors. (A-D) *nos-Gal4::VP16, UASp-GFP* driving expression. GFP, (germ cells) is in green, Hts (stains fusomes) and LamC (stains nuclear lamina) in red, and DAPI (nuclei) in blue. A-B: scale bar = 20 µM, C-D: scale bar = 200 µM. (A) *LacZ* control germaria with progressive stages of cysts and egg chambers budded off from the niche. (B) *EcR.B1* over-expression germaria where there is only cystoblast-like cells throughout the germaria. (C) Whole pairs of control ovaries with various stages off egg chambers and oocytes. (D) Whole pairs of ovaries over-expressing *EcR.B1* where there is no egg chambers or oocytes. (E) Bar graph showing percentage of tumorous ovarioles and formed egg chambers. Dots represent percent from each sample group, bars represent standard error, and sample size is at the bottom of each bar. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$.

Tagged EcR shows nuclear localization in germline tumors.

Nuclear localization of EcR is indicative of transcriptionally active protein (King-Jones & Thummel, 2005). To visualize EcR localization and spatial expression, we generated fluorescent tagged EcR proteins under UASz control (Figure 4). For EcR.B1 we added a mCherry protein to the N-termini and the C-termini. When overexpressing the N-terminal tagged EcR.B1 (*mCherry::EcR.B1*) proteins, germline tumors are generated at a frequency equivalent with the untagged protein (Figure 4A). We also see expression of the mCherry protein in all the germline tumors, localized predominately to the nucleus, and lesser amounts in the cytoplasm (Figure 4A). We suspect we see cytoplasmic localization due to the overabundance of the protein we expressed. The C-terminal mCherry (*EcR.B1::mCherry*) partially allowed egg chamber development, yielding germaria of an intermediate phenotype with lower tumor frequency and more egg chambers produced (Figure 4C). This protein also localized to the nucleus in both tumorous and non-tumorous germ cells (Figure 4B). This data suggests that addition of the C-terminal mCherry may have partially blocked access of ligand to the EcR ligand binding domain or caused a conformational change in the protein, altering its function.

EcR.B1 over-expression is sufficient to generate tumors only in GSCs and cystoblasts and only at high levels.

The *nos-Gal4::VP16* driver is expressed in GSCs and their differentiating daughters. We therefore considered whether germ cells at different stages of differentiation were equally competent to respond to excess EcR. Unfortunately, a GSC-specific Gal4 driver (that is not expressed in cystoblasts) is not available. In the absence of this tool, we drove *EcR.B1* over-expression in specific populations of undifferentiated cells and assay tumor frequency. (Figure

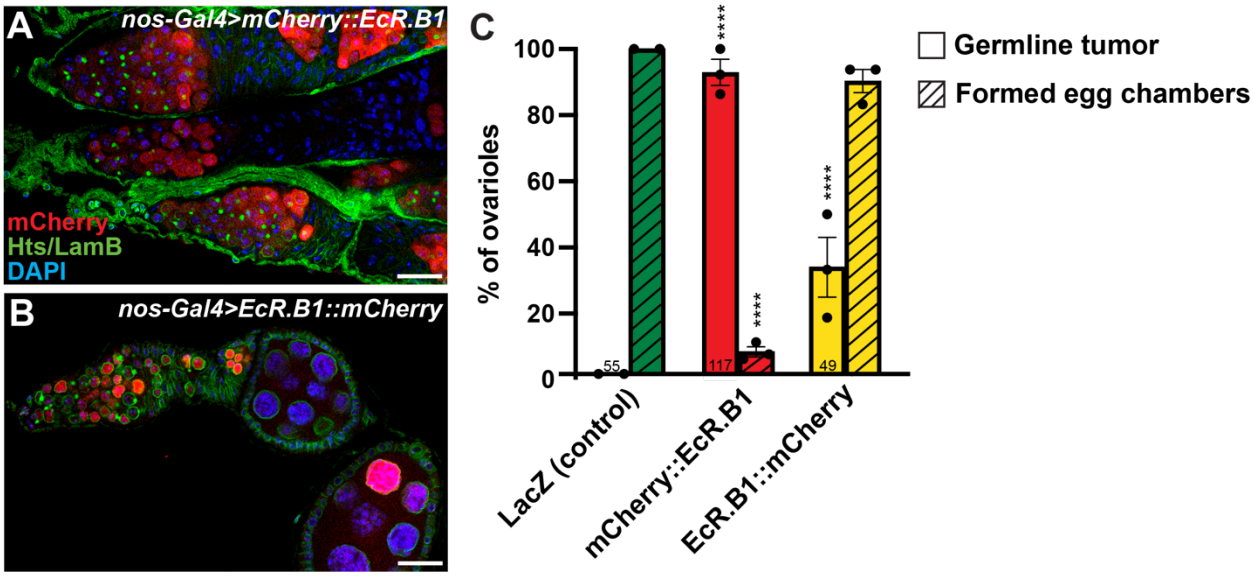

Figure 4: Tagged EcR shows nuclear localization in germline tumors. (A-B) *nos-Gal4::VP16* driving expression of mCherry tagged EcR.B1. Germaria stained for mCherry in red, Hts and Lamin B (LamB) in green, and DAPI in blue. Scale bar = 20 μM. (A) Over-expression of EcR.B1 with a N-terminal mCherry tag. mCherry signal is seen within and outside the nucleus. Cystoblast-like cells are present throughout the germaria. (B) Over-expression of EcR.B1 with a C-terminal mCherry tag. mCherry signal is detected within the nucleus. Cystoblast-like cells are present throughout the germaria, and egg chambers are produced from the niche. (C) Bar graph showing percentage of tumorous ovarioles and formed egg chambers. Dots represent percent from each sample group, bars represent standard error, and sample size is at the bottom of each bar. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$.

5). Using *otu-Gal4::VP16*, which drives expression in 16-cell cysts and developing egg chambers, we tested whether over-expression of EcR is sufficient to switch the fate of post-mitotic cells. However, over-expression of *EcR.B1* under *otu-Gal4::VP16* did not generate tumors or any noticeable issues with egg chamber development, likely since nurse cell and oocyte fates are already determined at these stages (Figure 5B).

Next, using *Bam-Gal4*, *EcR.B1* was overexpressed in early, mitotic germ cells (primarily the 4, 8, and early 16-cell cysts). Under this Gal4 control, we again have no tumor formation (Figure 5C). We also over-expressed *mCherry::EcR.B1* to confirm the spatial expression and see strong mCherry signal within the early germ cells (Figure 5D). To try expressing EcR in the earliest GSC daughter cells, we utilized *aub-Gal4*. This driver expresses in a mosaic pattern, occasionally weak GSC and cystoblasts expression and progressively stronger expression through the developing cysts. Over-expressing *EcR.B1* under *aub-gal4* there were no instances of germline tumors (Figure 5E). Altogether, restricting over-expression to the cysts and egg chambers is not able to generate germline tumors like overexpressing in all germ cells does. This indicates that over-expression is sufficient to alter cell fate only when expressed in the GSCs and cystoblast.

The level at which EcR is being over-expressed is also critical for the tumor phenotype. Using a germline driver that expresses *EcR.B1* at lower levels in all germ cells, *NGT-Gal4*, there was no tumor formation (Figure 5F). Although we do not have a quantitative measure on the level at which EcR.B1 is over-expressed, we can conclude that the titer of protein is pertinent for the promotion of an undifferentiated fate.

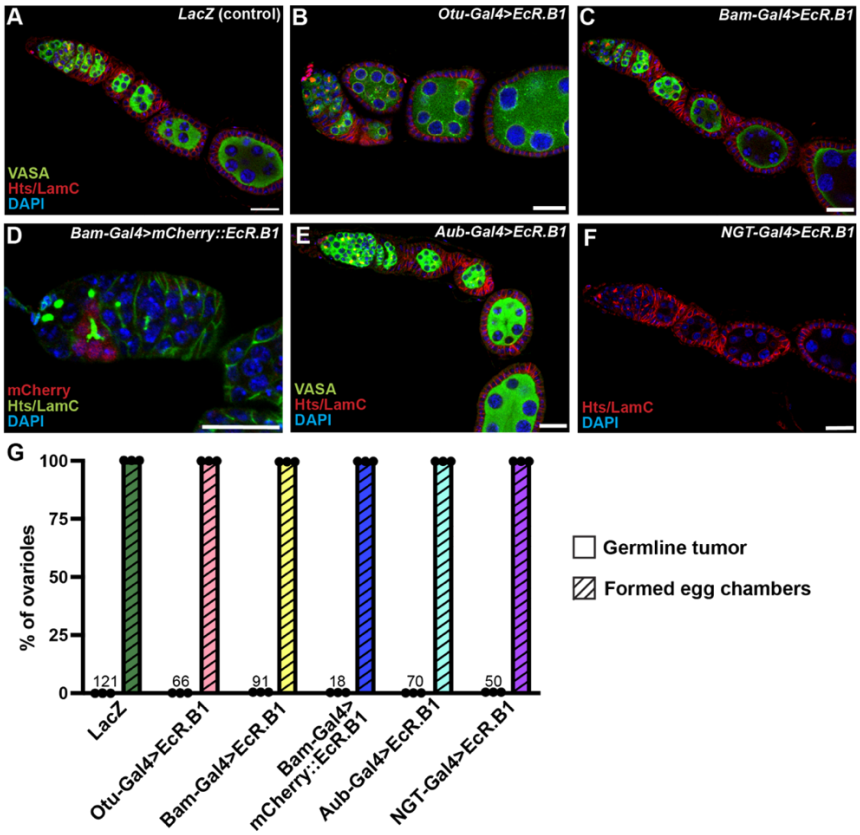

Figure 5: *EcR.B1* over-expression is sufficient to generate tumors only in GSCs and cystoblasts and only at high levels. (A-F) Different germline Gal4 drivers expressing *EcR.B1*. Scale bar = 20 μM. (A-C, E-F) Vasa stained in green, Hts and LamC in red, and DAPI in blue. (A) Control germaria expressing *LacZ* under *Bam-Gal4*, no tumors formed. (B) *Otu-Gal4* driving expression in post mitotic cells, no tumors formed. (C) *Bam-Gal4* driving EcR.B1 expression in early mitotic cells, no tumors formed. (D) *Bam-Gal4* driving expression of *mCherry::EcR.B1*. mCherry stained in red, Hts and LamC in green, and DAPI in blue. No tumors formed. (E) Aub-Gal4 driving expression in a mosaic pattern, no tumors formed. (F) *NGT-Gal4* driving low levels of *EcR.B1* expression in all germ cells, no tumors formed. (G) Bar graph showing percentage of tumorous ovarioles and formed egg chambers. Dots represent percent from each sample group, bars represent standard error, and sample size is at the bottom of each bar. Significance was tested using Fisher's exact test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$.

EcR.B1 dominant negatives reveal ligand binding facilitates tumor phenotype.

EcR can bind open chromatin in both the presence and absence of ecdysone (Uyehara et al., 2022). In the absence of ligand, in the model posited by Ashburner, EcR is thought to repress transcription, while ligand binding is thought to activate transcription. To test whether the tumor phenotype generated by over-expression of EcR reflected a ligand dependent function, we overexpressed forms of EcR that cannot activate transcription (Cherbas et al., 2003). Over-expression of $EcR.B1^{F645A}$, that can bind ligand but cannot activate transcription *in vitro*, did not generate germline tumors (Figure 6B). This indicates that the undifferentiated germline tumors caused by over-expression of *EcR.B1* is in an ecdysone dependent manner. Over-expression of one of the dominant negatives, $EcR.B1^{W650A}$, which cannot bind ligand or activate transcription, did generate germline tumors (Figure 6C). However, the phenotype is milder, as they form tumors less frequently than wildtype *EcR.B1* and similar to *EcR.B1::mCherry*. We speculate that this phenotype is not a result of loss of ligand binding activity, since the $EcR.B1^{DBL}$ that also contains the same amino acid substitution in addition to the F645A substitution, does not generate tumors (Figure 6D).

Increasing ecdysone levels suppresses tumor formation.

Given that $EcR.B1^{F645A}$ and $EcR.B1^{W650A}$ yielded different phenotypes, we asked whether levels of ecdysone influenced tumor formation in *EcR.B1* over-expression females. To increase levels of ecdysone present, we fed adult females over-expressing *EcR.B1* the active form of ecdysone, 20E, in their diet for five days (Figure 7). Supplemental ecdysone partially rescued the tumor phenotype, where we saw a decrease in frequency of tumors but did not see an increase in egg chambers formed (Figure 7 C and D).

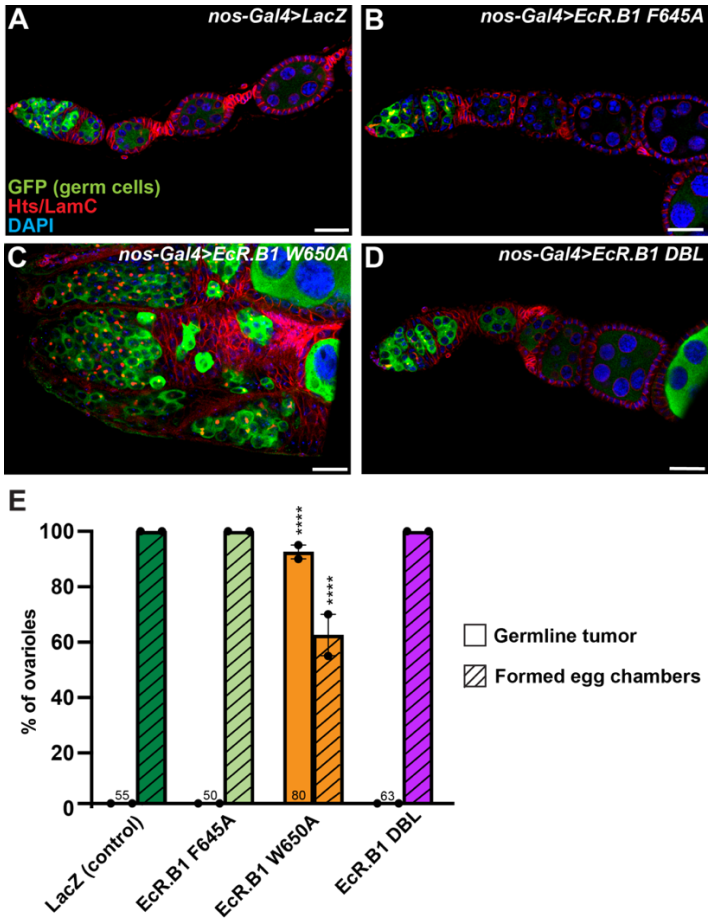

Figure 6: EcR.B1 dominant negatives reveal ligand binding facilitates tumor phenotype.

(A-D) *nos-Gal4::VP16, UASp-GFP* drove expression of EcR.B1 dominant negatives in all germ cells. GFP marks germ cells in green, Hts and LamC in red, and DAPI in blue. Scale bar = 20 µM. (A) Control germaria expressing *LacZ*. (B) Germaria over-expressing $EcR.B1^{F645A}$ with typical cyst and egg chamber progression. (C) Tumorous germaria over-expressing $EcR.B1^{W650A}$. (D) Germaria over-expressing dominant-negative $EcR.B1^{DBL}$ with typical cyst and egg chamber development. (E) Bar graph showing percentage of tumorous ovarioles and formed egg chambers. Dots represent percent from each sample group, bars represent standard error, and sample size is at the bottom of each bar. Significance was tested using Fisher's exact test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$

Upon mating and deposition of sex peptide in the male seminal fluid, females increase ecdysone biosynthesis; thus, unmated females have low titer of ecdysone (Ameku & Niwa, 2016). We therefore tested whether mating influenced tumor production. Female flies were kept unmated for five days and in those ovaries, there was no significant change in tumor formation or egg chamber production (Figure 7 E and F). This suggests that early germ cells respond to very low levels of ecdysone.

Understanding that ecdysone levels is an important switch for EcR's function in the germline, it became important to determine wildtype levels of ecdysone. Previous studies utilized EcR ligand sensors which act as ecdysone reporters (Kozlova & Thummel, 2002). These reporters, however, do not express in the germline, likely due to degradation by piRNAs (Ables, unpublished). We created a germline compatible EcR ligand sensor in which the constitutive germline promoter, *nanos*, should drive expression of a protein with the Gal4 DNA binding domain fused to the EcR hinge and ligand binding domain. When the protein binds ecdysone, it allows the Gal4 DBD to bind a UAS element and turn on expression of a reporter. Unfortunately, the reporter was not expressed in the germline and therefore could not be used as an evaluation of ligand availability.

Over-expression of EcR.A phenocopies EcR.B1.

It is plausible that the undifferentiated germ cell tumors caused by over-expression of EcR were a B1 isoform-specific phenotype. To over-express the A isoform, we also generated a UASz EcR.A transgene that had the equivalent early sequence termination in the ligand binding domain, *EcR.A* $^{\Delta C627}$. Over-expression of EcR.A phenocopied EcR.B1 and we see no difference

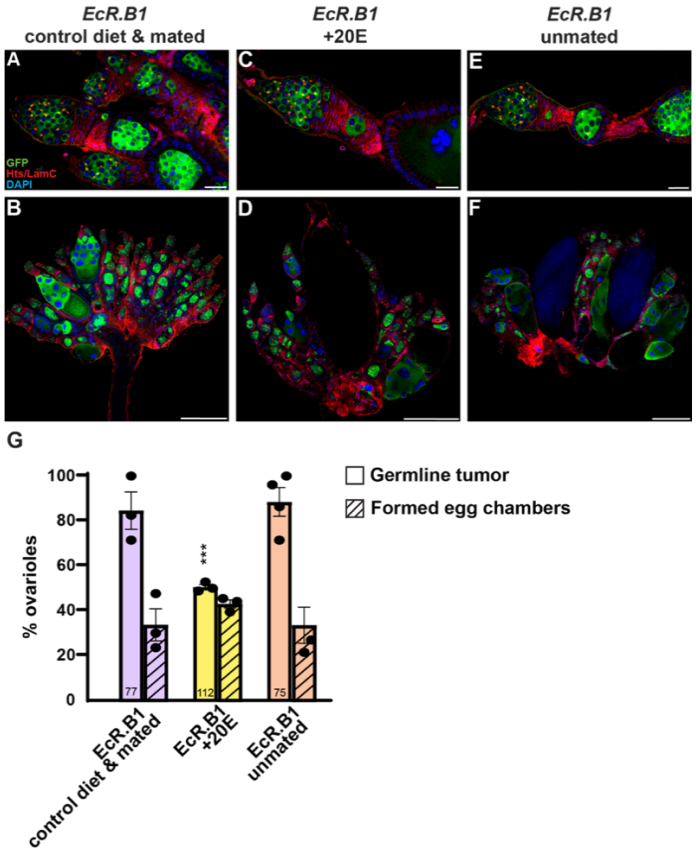

Figure 7: Increasing ecdysone levels suppresses tumor formation. (A-F) *nos-Gal4, UASp-GFP* driving over-expression of *EcR.B1*. GFP in green, Hts and LamC in red, and DAPI in blue. (A-B) Germaria and ovaries from flies that were maintained on a control wet yeast diet and mated. Germaria contain cystoblast-like cells throughout and egg chambers lack large nurse cell nuclei. (C-D) Germaria and ovaries from flies that were fed excess 20E in their diet and mated. Germaria contain later stages of cyst development and are less frequently tumorous. (E-F) Germaria and ovaries from flies that were fed a control diet and kept unmated. Germaria contain tumorous germ cells. (G) Bar graph showing percent of ovarioles with tumors and egg chambers. Dots represent percent from each sample group, bars represent standard error, and sample size is at the bottom of each bar. Significance was tested using Fisher's exact test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$.

in tumor formation or egg chamber development (Figure 8A). We conclude that both isoforms are sufficient to promote an undifferentiated fate.

We also found that the over-expression of mCherry tagged EcR.A constructs in all germ cells generated germline tumors at similar frequencies to the B1 tagged constructs. We found that N-terminal tagged EcR.A (*mCherry::EcR.A*) formed tumors at similar frequency to the untagged construct, and the C-terminal (*EcR.A::mCherry*) generated tumors at a lower frequency (Figure 8C and D). Both proteins had nuclear mCherry localization, indicative of transcriptionally active EcR.A.

We also attempted to express both mCherry tagged proteins in germ cells at a lower level using a different Gal4 driver, *NGT-Gal4::VP16*, to visualize nuclear localization. Expressing under this Gal4 driver we could not see strong enough mCherry signal to distinguish nuclear localization in the early germ cells and egg chambers (Figure 8 E and F). With this weaker expression we also did not generate germline tumors, supporting that high levels of EcR protein is necessary to for the promotion of an undifferentiated fate.

Although over-expression of EcR-B1 or A had no difference in phenotype, loss of function could reveal an isoform specific role. To do so, equivalent EcR.A dominant negatives that cannot bind ecdysone were generated into the UASz construct. The $EcR.B1^{F645A}$ equivalent, $EcR.A^{F617A}$ did not generate tumors (Figure 8G). The $EcR.B1^{W650A}$ equivalent, $EcR.A^{W622A}$, did however generate germline tumors (Figure 8H). Because all three dominant-negatives had no meaningful difference in phenotype from either isoform, we conclude that these genetic tools are not effective at discerning isoform-specific roles.

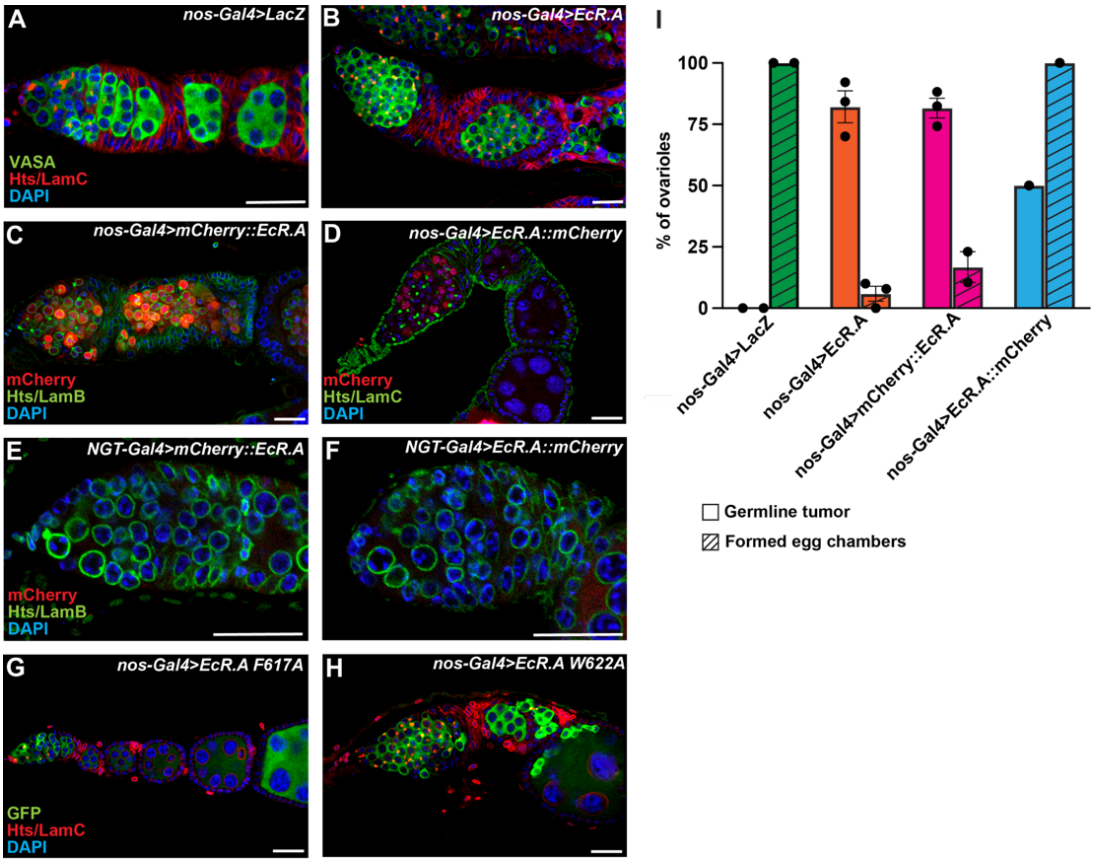

Figure 8: Over-expression of *EcR.A* phenocopies *EcR.B1*. (A-B) Germaria with *nos-Gal4::VP16* driving expression in the germ cells. Vasa which stains germ cells in green, Hts and LamC in red, and DAPI in blue. (A) *LacZ* control germaria with various stages of cyst development. (B) Over-expression of *EcR.A* where there are only cystoblast-like tumors. (C-D) *nos-Gal4::VP16* driving expression. mCherry in red, Hts and LamB in green, and DAPI in blue. (C) Over-expression of N-terminally tagged EcR.A, where mCherry localized to the nucleus and cytoplasm. Germline tumors were formed and we see no nurse cell nuclei. (D) Over-expression of C-terminally tagged EcR.A, where mCherry localized to the nucleus. There is some later stages of cyst development and egg chambers produced from the niche. (E-F) Weaker, germ cell driver *NGT-Gal4* driving expression. mCherry in red, Hts and LamB in green, DAPI in blue. (E) Expression of N-terminally tagged EcR.A at lower levels with no detectable signal in the

germline or tumor formation. (F) Expression of C-terminally tagged EcR.A at lower levels with no detectable signal in the germline or tumor formation. (G-H) *nos-Gal4, UASp-GFP* driving expression of EcR.A dominant-negatives. GFP which marks germ cells in green, Hts and LamC in red, and DAPI in blue. (G) Over-expression of $EcR.A^{F617A}$ in the germline, where cysts and egg chambers developed progressively. (H) Over-expression of $EcR.A^{W622A}$, where germline tumors are formed. (A-H) Scale bar = 20 μM. (I) Bar graph showing percentage of tumorous ovarioles and formed egg chambers. Dots represent percent from each sample group, bars represent standard error, and sample size is at the bottom of each bar. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$.

EcR over-expression causes GSC loss and stem-like tumors.

Although germ cells in *EcR* over-expression ovaries fail to differentiate properly to produce cysts, it is plausible that is due to failure of turning off their stem cell program. BMP signaling is a well characterized pathway necessary for ovarian germline stem cells. BMP ligands, decapentapalegic (dpp) and glass bottom boat (gbb), are secreted by the somatic cap cells and are received by the cell-surface receptors thick veins (Tkv) or Saxophone (Sax) and Punt (Xie & Spradling, 1998; Xie & Spradling, 2000;). Upon binding of dpp or gbb and the dimerization of the receptors, the type I receptors (Tkv or Sax) phosphorylate the transcription factor Mothers against decapentaplegic (Mad). Phosphorylated Mad (pMad) along with Medea, translocate to the nucleus and repress differentiation genes such as *bag of marbles* (*Bam*) (Chen & McKearin, 2003a; Song et al., 2004). After GSC division the cystoblast moves further away from the BMP source and no longer receives the signal to repress differentiation (Chen & McKearin, 2003b).

To identify if germline tumors express stem cell markers appropriately, we stained them with an antibody against phosphorylated Mad (Figure 9A). We were able to identify pMad positive stem cells correctly positioned within the niche in control and EcR.B1 over-expression (Figure 9B). However, in EcR.B1 overexpression we find that the average number of GSCs is decreased, indicating that increased levels of EcR also impacts self-renewal (Figure 9E).

Interestingly, in the EcR overexpression ovaries, pMad positive cells were also detected outside of the GSC niche. While BMP ligand is secreted from cap cells for the GSCs, the follicle cells of the mid-stage egg chambers are also a source of dpp protein (Rust et al., 2020). In the occasion where the EcR overexpression ovaries formed egg chambers with nurse cells, smaller,

pMad-positive germline tumors could be identified (Figure 9 C and D). This shows that the EcR germline tumors remain competent to respond to dpp signals.

Stem-like cells could be derived from over-proliferative GSCs or mitotically active tumor cells. To see if the tumors are still mitotically active, we stained ovaries for phospho-histone 3 (PHH3), which positively marks cells during mitosis, and EdU, which labels cells during S phase. At five days after eclosion, we find that germ cells throughout the ovary are mitotically active (Figure 9H). This suggests that the tumors developed from symmetric and complete divisions of the stem-like tumor cells.

Germline tumors caused by EcR.B1 do not express differentiation markers.

Other genes expressed in the BMP signaling pathway can be indicative of tumor cell differentiation. Daughters against dpp (Dad) is a protein induced by dpp signaling and is found typically in control GSCs and cystoblast (Casanueva & Ferguson, 2004) (Figure 10J). Using a *Dad-LacZ* reporter, we find β-gal expression in the GSCs of *EcR.B1* over-expressing germaria. Using a *Bam-GFP* reporter line, we can identify if these tumor cells turn on one of the critical differentiation promotion genes. In control germaria, GFP can be detected in 2 and 4-cell cysts (Figure 10G). In *EcR.B1* over-expression, majority of tumor cells are GFP negative, but there is sporadic expression of GFP in cells throughout the ovary (Figure 10H). This expression indicates that these germ cells are not differentiating properly due to lack of proper gene expression.

Outside of the canonical BMP signaling pathway, other proteins are also critical for the promotion of early germ cell differentiation. Rbfox1 is an RNA binding protein that is specifically upregulated in the cystoblast to repress pumilio translation (Carreira-Rosario et al., 2016). Using an antibody against Rbfox1, protein can be levels are high enough to be visualized

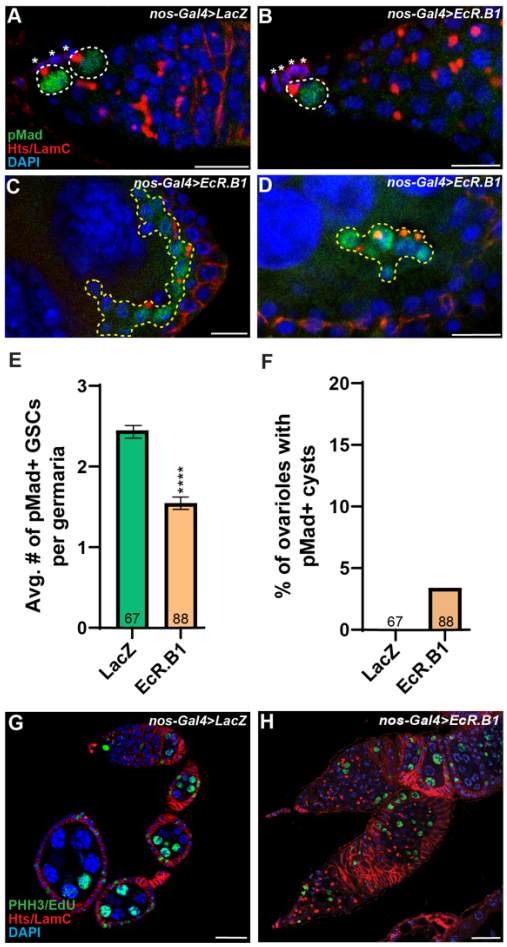

Figure 9: *EcR* over-expression causes GSC loss and stem-like tumors. (A-D) Ovaries stained for the stem cell marker, pMad. Asterisks mark cap cells, dotted white lines outline GSCs, and dotted yellow lines outline pMad positive tumors. pMad in green, Hts and LamC in red, and DAPI in blue. Scale bar = 10 μM. (A) *LacZ* control germaria. (B) *EcR.B1* over-expression germaria. (C-D) *EcR* over-expression tumors in egg chambers. (E-F) Ovaries stained for PHH3 and EdU in green, Hts and LamC in red, and DAPI in blue. Scale bar = 20 μM. (E) *LacZ* control ovariole. (F) *EcR.B1* over-expression. (G) Bar graph showing average number of pMad+ GSCs per germaria. Significance measured using student's t-test. (G) Bar graph of percent of ovarioles with pMad+ cysts. Bars represent standard error, and sample size is at the bottom of each bar. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$.

starting around the 4-cell cyst stage in controls (Figure 10D). In *EcR.B1* tumors, Rbfox1 antibody is not detected in the germ cells (Figure 10E). Other differentiation markers such as, Orb, an oocyte-specific protein, can also indicate if tumors have terminally differentiated (Barr et al., 2019) (Figure 10A). In *EcR.B1* over-expression germ cells, Orb protein was not detected (Figure 10B).

EcR.B1 and Tkv^{ACT} tumors reveal striking transcriptional similarity.

Comparing our *EcR.B1* over-expression phenotype to other known tumor phenotypes can also provide insight into germ cell fate determination. Manipulation of the BMP signaling pathway in the germline also generates germline tumors. In *Bam* mutant ovaries, undifferentiated germ cell tumors are produced because of the loss of the ability for cysts to differentiate (McKearin & Spradling, 1990; McKearin & Ohlstein, 1995). Like *Bam* mutants, over-expressing a constitutively active Tkv protein (Tkv^{ACT}) also generates stem-like germline tumors (Casanueva & Ferguson, 2004) (Figure 11B). The Tkv^{ACT} protein constituitively phosphorylates Mad irrespective of ligand binding, promoting repression of differentiation genes. Like EcR over-expression, this leads to an over-production of stem-like undifferentiated germ cells (Figure 10 C, F, I, L).

To identify the transcriptional changes of *EcR.B1* over-expression germ cell tumors and how it compares to overactive BMP signaling, we utilized single-cell RNA-sequencing (scRNA-seq). Several labs have been successful using this technique in the ovary and been able to resolve both germ and somatic population transcriptional profiles (Slaidina et al., 2021; Rust et al., 2020; Jevitt et al., 2020). In this approach, we anticipated that using wild-type ovaries as a comparison for the EcR.B1 over-expression sample would not permit sufficient resolution, since the GSCs

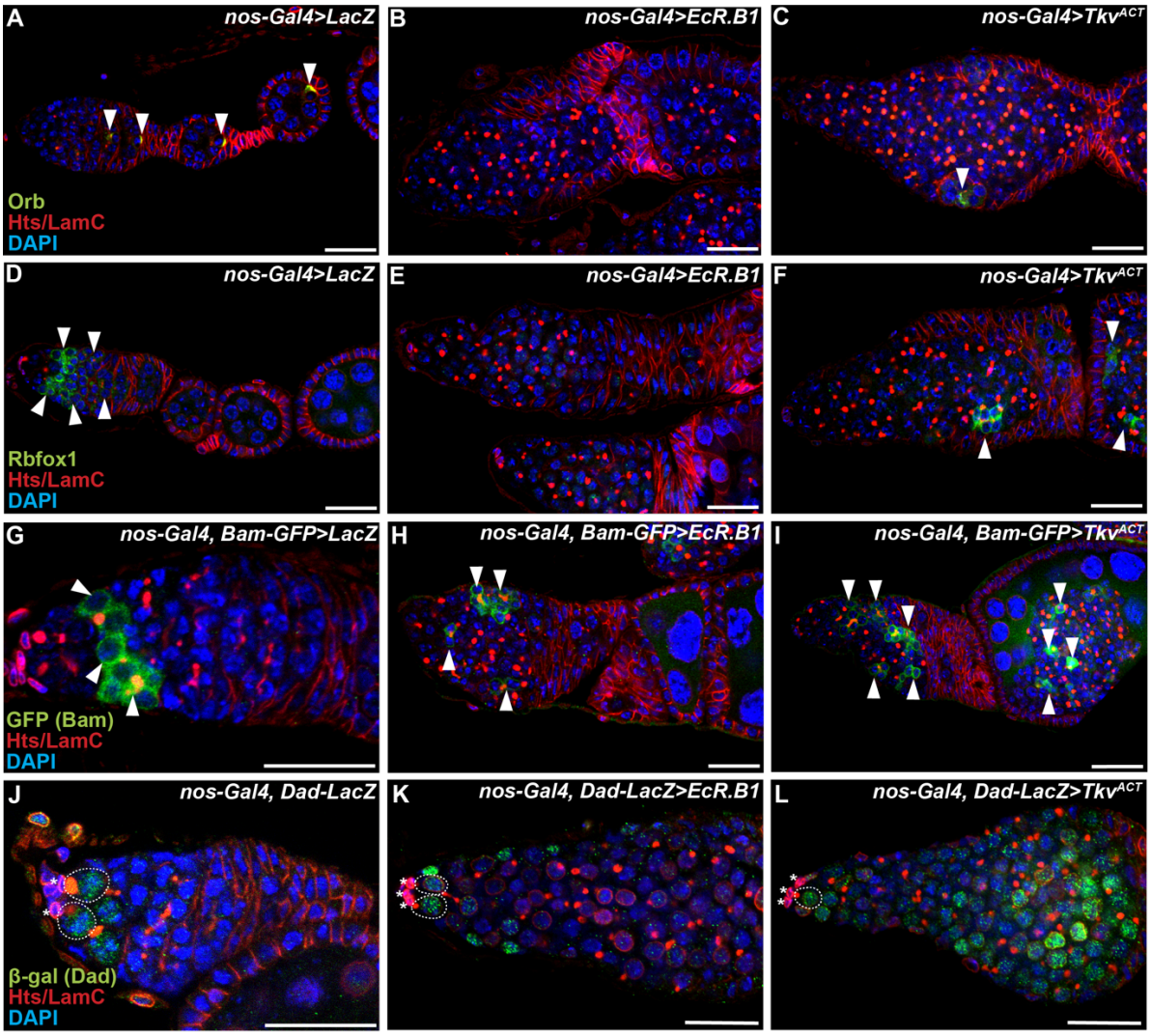

Figure 10: Germline tumors caused by EcR.B1 do not express differentiation markers. (A-C) Germaria stained for the Orb in green, Hts and LamC in red, and DAPI in blue. Arrowhead points to oocyte marked by orb. Scale bar = 20 μM. (D-F) Germaria stained for RbFox1 in green, Hts and LamC in red, and DAPI in blue. Arrowhead points to RbFox1+ cysts. (G-H) Over-expression driven by *nos-Gal4, Bam-GFP*. GFP in green, Hts and LamC in red, and DAPI in blue. Arrowheads point to Bam+ cysts. (J-L) Over-expression driven by *nos-Gal4, Dad-LacZ*. β-gal in green, Hts and LamC in red, and DAPI in blue. Asterisks mark the cap cells and Dad+ GSCs are outlined in white dashed lines.

and cystoblasts are a small fraction of total cells. Instead, we opted to compare *EcR.B1* over-expression to Tkv^{ACT} over-expression since both enrich for early germ cells.

Pooling both the EcR and Tkv samples and projecting them into a UMAP, we visualize two main groups from 16 clusters (Figure 10C). The lower, strawberry-shaped group was highly enriched for germ cell markers *nanos* and *Vasa* (clusters 2,3,4,5,6, and 14) (Figure 10D). Whereas the upper, flower-shaped group enriched for various somatic cell markers such as *traffic jam*, *fasclin3*, and *eyes absent*.

We then identified six germ cell subpopulations (Figure 10E). In the tSNE spatial projections, the EcR.B1 and Tkv^{ACT} sample are minimally overlapped from one another but share the same six subpopulations in varying proportions (Figures 10 F-K). Looking at the expression of early germ cell/GSC markers such as *benign gonial cell neoplasm* (*bgcn*) or *meiotic p26* (*mei-p26*) we see consistent expression in all clusters (Li et al., 2012; Ohlstein et al., 2000) (Figure 10 L-M). It cannot be determined with confidence which subpopulation of the germ cells are the true stem cells because GSCs do not have an enriched, discrete transcriptional identifier.

Looking at the germ cells in bulk, there are very few genes significantly and highly upregulated or highly downregulated comparing these two over-expression samples. One highly upregulated gene, in the EcR.B1 sample is an uncharacterized gene, *CG43134* (Log2 fold change = 3.65, p = 8.43 e-6). Although it is a relatively small protein at 82 amino acids, there are no predicted functions, and we cannot draw conclusions on how this is involved in germ cells. *Female sterile (1) k10* (*Fs(1)k10*) is a gene that is downregulated in the EcR.B1 sample (Log2 fold change = -3.96, p = 1.03 e-40). *Fs(1)k10* has been previously implicated in oogenesis and dorsoventral patterning, as it is necessary for *gurken* mRNA localization in the developing

oocyte (Serano et al., 1995). Interestingly, *Rps19b* is downregulated in the EcR.B1 sample. Rps19b is a ribosomal protein enriched in the GSCs and early cysts and modulates the translation of the differentiation factor, Rbfox1 (McCarthy et al., 2022). The downregulation of this ribosomal protein could be one of the indirect or direct targets EcR acts upon to restrict differentiation.

Analysis of a germline tumor transcriptome.

While over-expressing either EcR.B1 or Tkv^{ACT} generates early germ cells, there is variability in the germ cells produced. Looking at differential expression in the six germ cell subclusters, we see discrete expression across the clusters indicating that EcR.B1 or Tkv^{ACT} germline tumor are heterogenous with some variability in identity (Figure 12A). Cluster 14 shows the most distinct transcriptional profile. Interestingly, the most-upregulated genes such as *sidestep* (*side*), *pale* (*ple*), and *Rab3-GEF*, are all enriched in neurons (Fly Cell Atlas). However, it is not likely that Cluster 14 cells are neurons due to the absence of stereotypical neuronal markers such as *N-cadherin*, *elav*, or *nSyb*. This cluster is also enriched for two Snail family transcription factors *escargot* (*esg*) and *scratch* (*scrt*) which work to negatively regulate Notch-mediated neuronal cell fate commitment (Remat et al., 2016). Although dispensable for spermatogenesis, escargot is a male sex-specific GSC marker (Streit et al., 2002). While there is no evidence that these neuronal-associated genes have a role in the typical germline, they strike curiosity about their potential role in differentiation and regulation by EcR or BMP signaling.

With the high level of similarity between the two tumor transcriptomes, it is plausible that these signaling pathways regulate one another. We know by over-expressing Tkv^{ACT}, we increased the level of its expression; but, when we compare it to level of *Tkv* expression in the

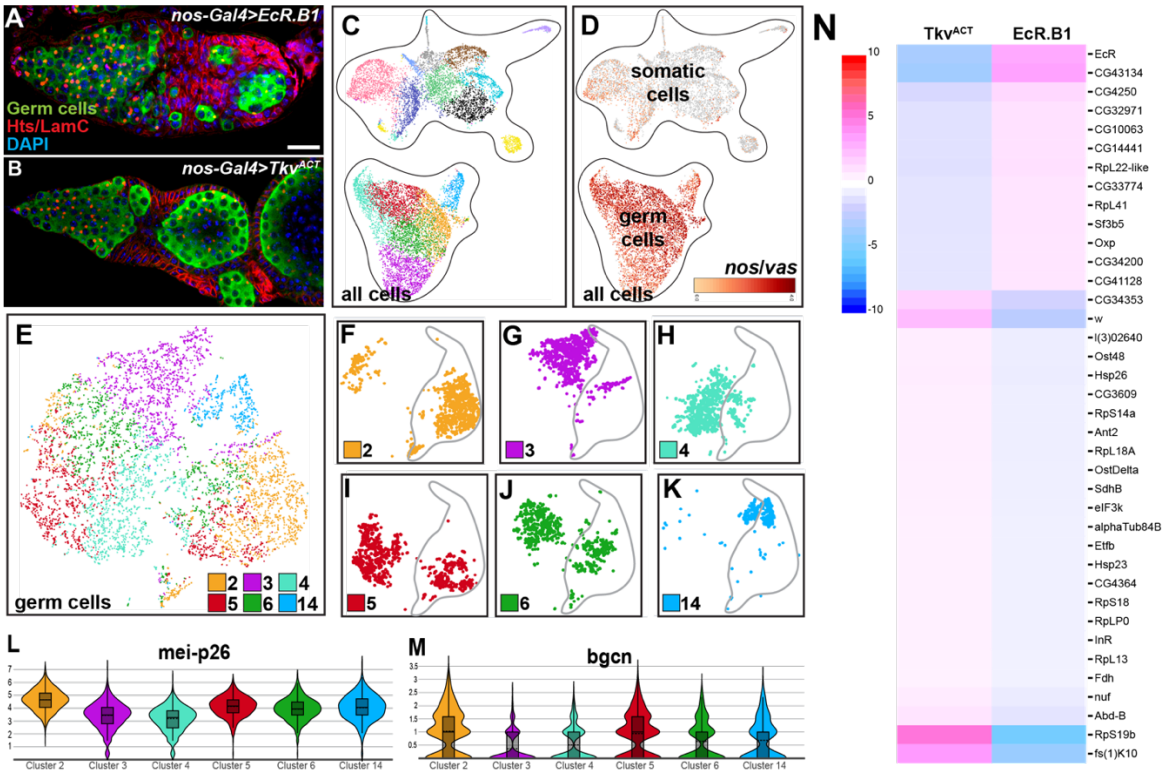

Figure 11: EcR.B1 and Tkv^{ACT} tumors reveal striking transcriptional similarity. (A-B) Images of germline tumors with germ cells in green, Hts and LamC in red, and DAPI in blue. Scale bar = 20 µM. (A) *EcR.B1* over-expression tumors. (B) *Tkv^{ACT}* over-expression tumors. (C) UMAP projection of all cells from both EcR.B1 and Tkv^{ACT} samples. Each color denotes a different cluster identified, and each dot represents one cell. (D) Germ cells, identified by *nanos* and *Vasa* expression (red). (E) tSNE project of only germ cells from both EcR.B1 and Tkv^{ACT} samples. (F-K) tSNE projections of each germ cell subcluster from both samples. EcR.B1 cells outlined in grey, and Tkv^{ACT} cells are un-outlined. (L) Violin plot of *mei-p26* expression in the six germ cell subclusters. In the box plot, the bottom line represents the lower 25% and the upper represents the upper 25%, the solid line denotes the median, and the dashed line denotes the mean of the cells in the cluster. (M) Violin plot of *bgcn* expression. (N) Heatmap comparing Log2Fold change of gene expression in Tkv^{ACT} and EcR.B1 germ cells. Pink = high expression and blue = low expression.

EcR.B1 sample there is no statistical difference in expression (Figure 12B). This indicates that by over-expressing EcR.B1, we also increased *Tkv* expression. This could be a mechanism by which these signaling pathways interact.

Knowing ecdysone levels are important for the EcR.B1 over-expression phenotype, it is important to look for evidence of germ cells synthesizing their own ecdysone. There are ten enzymes that belong to the Halloween gene family that convert dietary cholesterol to 20E (Rewitz et al., 2006). Looking at six of the Halloween genes (*phantom, shadow, shade, neverland, disembodied*, and *spooky*) we found no expression in the germ cells (Figure 12C). This is not surprising, as other transcriptomic analysis of the ovarian germ cells have also found no evidence of early germ cell ecdysone biosynthesis.

Canonical EcR targets were also identified in the germ cell clusters from both EcR.B1 and Tkv^{ACT} samples. mRNA of the orphan nuclear receptor, *Ftz-f1*, was found in all six subclusters (Figure 12F). Ftz-f1, an orphan nuclear receptor, has a characterized role in follicle cells for egg chamber survival and has enhancer activity in mid-stage egg chamber germ cells but does not have a described role in early germ cells (Beachum et al., 2021, McDonald et al., 2019). *E75* had more discrete expression and was only found in clusters 2 and 14 (Figure 12E). Unexpectedly, an ecdysone inducible protein, *E63E*, was expressed in all six subpopulations (Figure 12G). *E63E* encodes a cyclin dependent kinase that interacts with Cyclin Y (Stowers et al., 2000; Liu & Finley, 2010). While the mechanisms by which E63E and Cyclin Y function are not well understood, they are both necessary for developmental processes across the lifespan (Liu & Finley, 2010). For these three canonical targets, their expression is the highest in clusters 2 and 14 which are also the clusters with the highest *EcR* expression. From this analysis, we predict that *Ftz-f1, E75*, and *E63E* are direct targets of EcR.

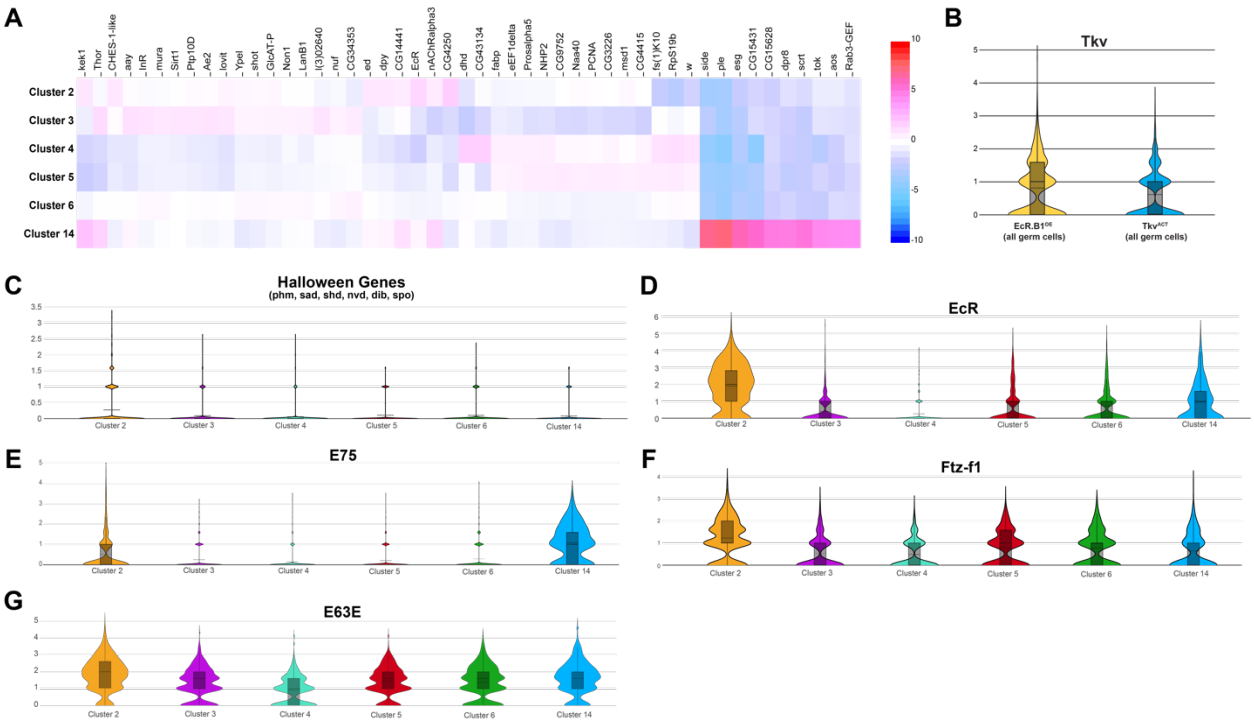

Figure 12: Analysis of a germline tumor transcriptome. (A) Heatmap of Log2Fold change in gene expression between germ cell subclusters. Red = high expression and blue = low expression. (B) Violin plot comparing *Tkv* expression from the two samples. In the box plot, the bottom line represents the lower 25% and the upper represents the upper 25%, the solid line denotes the median, and the dashed line denotes the mean of the cells in the cluster. Between EcR.B1 and Tkv^{ACT} samples, there is no significant difference in *Tkv* expression. (C) Violin plot of six Halloween Genes: *phantom* (*phm*), *shadow* (*sad*), *shade* (*shd*), *neverland* (*nvd*), *disembodied* (*dib*), and *spooky* (*spo*), where we see no expression in the six germ cell subclusters. (D) Violin plot of *EcR* expression, where there is expression in all six clusters, but highest in clusters 2 and 14. (E) Violin plot of *E75* expression, where it is expressed in clusters 2 and 14. (F) Violin plot of *Ftz-f1* expression in all six subclusters. (G) Violin plot of *E63E* expression in all six subclusters.

Loss of EcR mRNA causes GSC loss in an autonomous manner.

If EcR over-expression caused an increase in stem-like fate, we predicted that loss of EcR would decrease stem fate and GSC maintenance. GSC loss had been observed in *EcR* global mutants, but it had not been demonstrated in a germ cell autonomous manner (Konig et al., 2011, Ables & Drummond-Barbosa, 2010; Morris & Spradling, 2012). We drove expression of EcR RNAi 6-4 in only the germ cells and found equivalent numbers of GSCs in *EcR*-depleted and driver-only germaria at eclosion (Figure 13C). However, over time the average number of GSCs decreased in EcR-depleted germaria and was significantly lower than control at 3, 6, and 10 days after eclosion (dae) (Figure 13C). This demonstrates that EcR is necessary in GSCs autonomously for their self-renewal.

EcR is necessary for cyst encapsulation.

Interestingly, we also found that loss of *EcR* mRNA in the germline resulted in encapsulation defects of 16-cell cysts in regions 2B and 3. In these regions, follicle cells should extend between adjacent cysts and zipper them into an egg chamber as they exit the niche. In EcR knockdown, there was a lack of follicle cells partitioning cysts, causing cyst collisions which became most prominent at 6 days after eclosion (Figure 14D). Even when cysts weren't captured in a collision state, they had a shortened distance between adjacent cysts compared to the control (Figure 14H). Lastly, it was observed that the shape of the region 3 cysts in the EcR knockdown had a flattened shape rather than the stereotypical globular which was measured by an aspect ratio. At 0-1 and 3 dae the average aspect ratio was significant but became consistent with the control at later time points (Figure 14L).

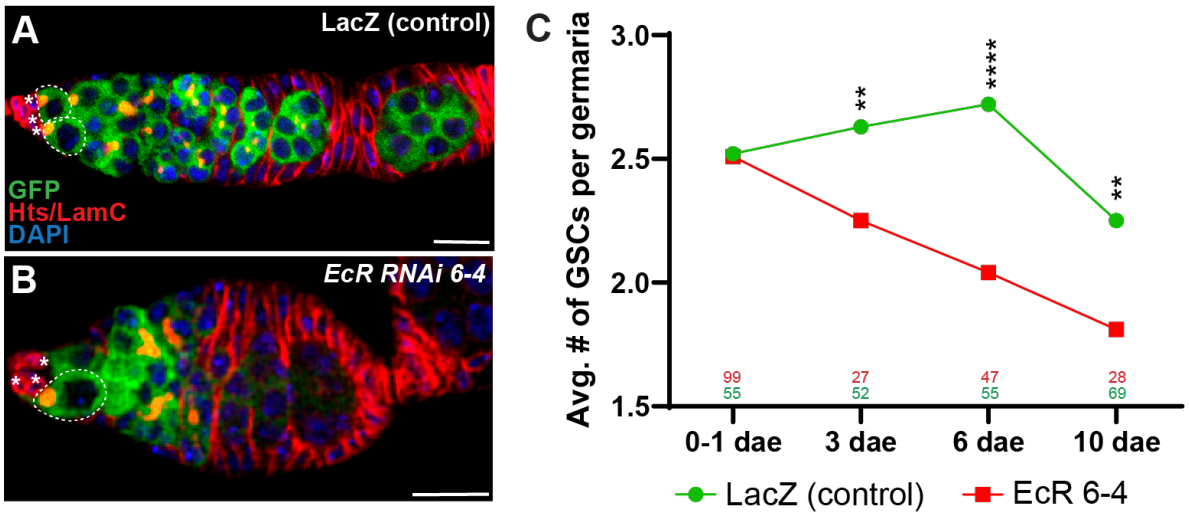

Figure 13: Loss of *EcR* mRNA causes GSC loss. (A-B) *nos-Gal4::VP16, UASp-GFP* driving expression of *EcR RNAi 6-4*. Asterisks mark cap cells and GSCs are outlined in white dashed lines. GFP in green, Hts and LamC in red, and DAPI in blue. (A) *LacZ* control germaria. (B) *EcR RNAi* germaria. (C) Line graph showing the average number of GSCs at four time points. In the *EcR RNAi* samples, we see a significant decrease in the average number of GSCs compared to the control at all time points except 0-1 dae. Sample size is listed above each time point. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 (Student's T-test).

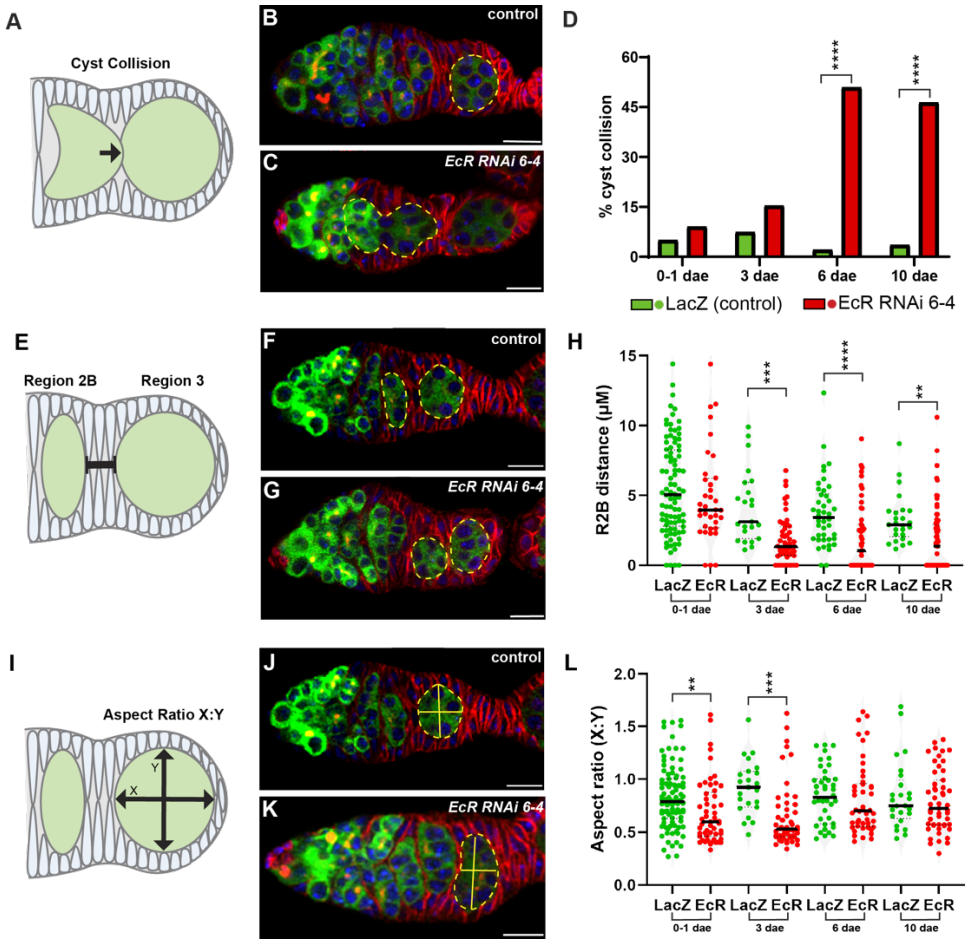

Figure 14: EcR is necessary for cyst encapsulation. *EcR RNAi 6-4* was driven in all germ cells using *nos-Gal4::VP16, UASp-GFP*. Samples were stained with GFP in green, Hts and LamC in red, and DAPI in blue. Yellow dashed lines outline region 2B/3 cysts. Scale bars represent 10 µM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$. (A) Diagram of cyst collision. (B-C) Germariums of *LacZ* control and *EcR RNAi 6-4* germaria. (D) Percent of germariums with cyst collision over time, significance tested using Fisher's exact test. (E) Diagram of region 2B/3 measurement. (F-G) Germariums of control and *EcR RNAi 6-4* (H) Violin plot of region 2B/3 distance, bar represents mean, significance tested using student's T-test. (I) Diagram of cyst aspect ratio. (J-K) Germariums of control and *EcR RNAi 6-4* (P) Violin plot of region 3 cyst aspect ratios, bar represents mean, significance tested using student's T test.

EcR is required in an ecdysone dependent manner in 16-cell cysts for encapsulation.

When EcR.B1 dominant negatives were expressed in the germ cells those germaria had encapsulation defects resembling *EcR RNAi 6-4*. Looking at *EcR.B1*^{F645A} and *EcR.B*^{DBL}, that did not generate tumors, there was a significant frequency of cyst collisions in the 2B/3 region (Figure 15H). While loss of EcR showed that it is necessary for encapsulation, this data shows that this role is ecdysone dependent.

We were also curious if there was a spatial requirement for EcR for proper encapsulation. Utilizing a different Gal4 driver, *Bam-Gal4*, that expresses in the early mitotic cysts we also found that *EcR.B1*^{F645A}, *EcR.B1*^{DBL}, and *EcR.B1*^{W650A} cause did not cause an increase in cyst collisions (Figure 15H). This suggests that EcR is dispensable in early mitotic cysts for encapsulation and is necessary in the 16-cell cysts.

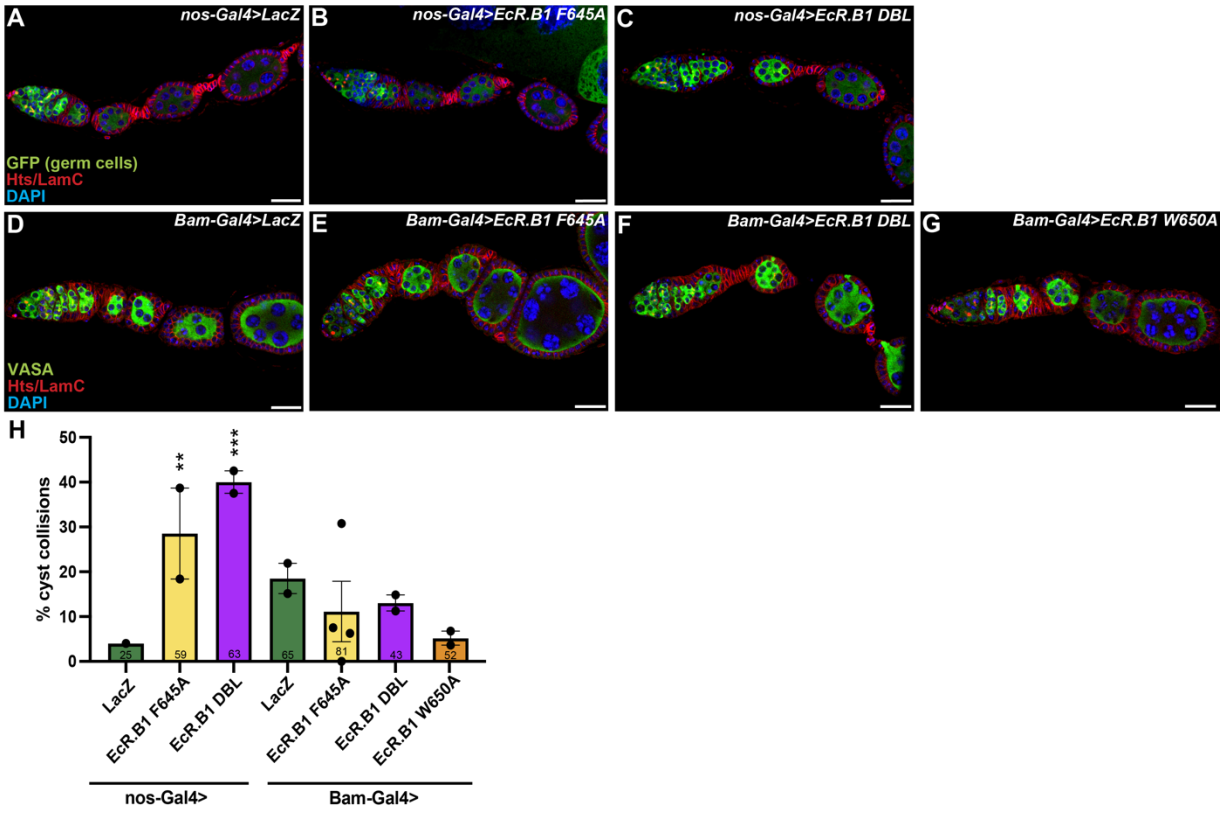

Figure 15: EcR is required in an ecdysone dependent manner in 16-cell cysts for encapsulation. (A-C) *nos-Gal4, UASp-GFP* driving expression. (A) *LacZ* control germaria. (B) $EcR.B1^{F645A}$. (C) $EcR.B1^{DBL}$. (D-G) *Bam-Gal4* driving expression in early mitotic cells. (D) *LacZ* control germaria. (E) $EcR.B1^{F645A}$. (F) $EcR.B1^{DBL}$. (G) $EcR.B1^{W650A}$. (A-G) Scale bar = 20 μM. (H) Bar graph showing percentage of germaria with cyst collision under *nos-Gal4* and *Bam-Gal4* control. Dots represent percent from each sample group, bars represent standard error, and sample size is at the bottom of each bar. Significance measured using Fisher's Exact test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$.

DISCUSSION

EcR is necessary autonomously for GSC self-renewal

Depleting *EcR* mRNA in the germline revealed that EcR is necessary for GSC self-renewal autonomously. Further investigation on GSC maintenance over time using the dominant negatives, *EcR.B1 F645A* and *EcR.B1 DBL*, could determine if EcR is required in an ecdysone-dependent role. However, over-expressing EcR that can bind ecdysone and activate transcription also generated a reduced number of GSCs at a stagnant time point. In this model, EcR is necessary for GSC self-renewal, but excess EcR promotes an undifferentiated fate in both the GSC and daughter cell. Together, these data reveal that the balance of EcR is necessary for GSC maintenance.

EcR is necessary and sufficient in GSCs and cystoblasts for promoting an undifferentiated fate

Driving over-expression of EcR.B1 resulted in undifferentiated, stem-like germ cell tumors. Although we were able to identify transcriptional changes of EcR in the germline by scRNA-seq, we cannot conclude which of these genes are direct targets. Using CUT&RUN to identify the sequences of the genome where EcR is binding, we could identify which of these genes are being upregulated in EcR.B1 over-expression using the mCherry tagged line. This could also reveal EcR enhancer sequences that are unique to the germline. CUT&RUN could also identify if EcR directly regulates the three upregulated canonical EcR targets (*Ftz-f1*, *E75*, and *E63E*). With the capabilities to build germline-compatible tools, it would be interesting to look at each of these canonical targets independently to see how they modulate cell fate. If EcR

is acting indirectly on germ cell differentiation through one of these targets, we could recapitulate the tumor phenotype by over-expressing them singularly.

Importantly, this role is dependent on ecdysone binding and ability to transactivate. Over-expressing EcR proteins that can bind ecdysone but cannot transactivate, EcR.B1^{F645A} or EcR.B1^{DBL}, did not generate germline tumors. However, over-expression of EcR.B1^{W650A} did generate tumors similar to EcR.B1$^{\Delta C655}$. It is unclear why this protein appears to not work as a dominant negative in the germline, when we find that it is sufficient to knockdown EcR in the lethality screen. The ligand affinity for the F645A and W650A proteins have only been tested *in vitro* (Cherbas et al., 2003). Further *in vivo* analysis on these proteins ability to bind enhancer sequences, bind ecdysone, and transactivate could discern their function.

We found that while the amount of EcR protein is important for controlling differentiation, so is the amount of available ligand. Feeding supplemental ecdysone in EcR.B1 over-expression females decreased the frequency of tumors. We had also found no evidence of these germ cells synthesizing their own ecdysone. It has been suggested that somatic escort cells or late stage follicles are a local source of ecdysone production (Xi et al., 2020). A local source of ecdysone could add another level of regulation of EcR in the germline, and the importance of germline-somatic communication.

EcR and BMP signaling may work together to control germ cell fate

From the transcriptional analysis of EcR over-expressing germ cells, it can be hypothesized that EcR may directly target components of the BMP signaling pathway to promote a stem-like fate. If knocking down a BMP component in an EcR over-expression is able to rescue the phenotype we could discern whether EcR is upregulating BMP signaling.

Another hypothesis is that EcR is opportunistically binding the chromatin landscape of the stem-like tumors and perpetuating their cell fate. In the ovary, GSCs are euchromatin rich while the differentiating daughter cells become increasingly rich for heterochromatin (Pang et al., 2023). It is likely that excess EcR is binding these open chromatin regions in the stem cells and upregulating genes promoting GSC fate. This is equally plausible for the BMP transcriptional regulator, pMad. We could identify if this is the case by doing an ATAC-sequencing analysis on EcR.B1 and Tkv^{ACT} over-expression germ cells.

EcR is necessary for proper encapsulation

Loss of EcR mRNA or EcR's transactivating function resulted in encapsulation defects. Encapsulation of cysts requires coordination between the germline and soma, but little is known about the mechanisms of this communication. Interestingly, ecdysone signaling is necessary specifically in the somatic cells for encapsulation and specification of primordial germ cells (Morris & Spradling, 2012; Gancz et al., 2011). While this highlights the importance of ecdysone signaling in both germ and somatic cells, more work is needed to understand how follicle cells and cysts interact for follicle formation to identify the mechanism of EcR in this process.

Understanding the role of ecdysone in the germline is of large developmental importance but had yet been investigated directly. In this study, we described an autonomous and direct role for the ecdysone receptor in GSC self-renewal and promotion of differentiation. Ecdysone signaling has been known to be necessary for GSC maintenance and using new germline-compatible tools to knockdown and over-express EcR we found that this is an autonomous role. We find a novel role for EcR in promoting an undifferentiated fate in GSCs. Germline tumors

formed from over-expressing EcR have a high level of similarity transcriptionally and phenotypically to over-expression of the BMP receptor, Tkv. Additionally, we find that loss of *EcR* or its transcriptional activation results in encapsulation defects. These data underscore the importance of understanding the role of hormones on stem cell differentiation that is imperative for oogenesis.

MATERIALS AND METHODS

Drosophila strains and husbandry

Flies were kept at 22-25°C on a standard diet of cornmeal/molasses/yeast/agar. Progeny were collected within 24 hours of eclosion and maintained at 25°C on standard medium supplemented with wet yeast paste for 5 days (changed daily) prior to ovary dissection.

Construction of EcR over-expression and dominant negative transgenics

To generate EcR.B1 over-expression and dominant-negative constructs, the coding domain sequence of *ΔC655 (EcR.B1)*, *EcR.B1 F645A*, *EcR.B1 W650A*, and *EcR.B1 DBL* (from Cherbas et al., 2003) were amplified out of *pUC57 mini* plasmids (GenScript) and cloned into the BamHI site of the pUASz-1.0 plasmid using Takara's In-fusion Snap Assembly Cloning. Plasmid sequences were verified via nanopore at Plasmidsaurus. Plasmid DNA was injected into embryos by BestGene and integrated into the *attp40* locus.

The CDS to over-express EcR.A was designed to have the same early termination as the *EcR.B1 ΔC655* CDS in the conserved ligand binding domain (*EcR.A ΔC627*). The same 3 dominant negatives were also designed to have the equivalent amino acid substitutions- *EcR.B1 F645A = EcR.A F617A*, *EcR.B1 W650A = EcR.A W622A*, *EcR.B1 DBL = EcR.A DBL*.

The *EcR.A* CDS was amplified out of a *pUC57 mini* plasmid (GenScript) and integrated into *pUASz 1.0* at the BamH1 site by In-fusion cloning. The A isoform dominant negatives were cloned by Site Directed Mutagenesis (SDM) in the *pUASz 1.0 EcR.A deltaC627* plasmid. Plasmid sequences were verified via nanopore at Plasmidsaurus. Plasmid DNA was injected into embryos by BestGene and integrated into the *attp40* locus.

Construction of EcR mCherry tagged transgenics.

EcR and *mCherry* CDS were amplified out of *pUC57* vectors and cloned into the BamHI restriction site of *pUASz 1.0* using Takara In-fusion cloning. Plasmid sequences were verified via nanopore at Plasmidsaurus. Plasmid DNA was injected into embryos by BestGene and integrated into the *attp40* locus.

Ovary dissection and immunostaining

For standard protocol- ovaries were dissected into Grace's insect medium and fixed in cold 5% paraformaldehyde in Grace's for 13 minutes at room temperature. Samples are rinsed with 0.1% phosphate buffered saline with Triton X-100 (PBST) and washed for 10 minutes three times, then permeabilized with 0.5% PBST for 20 minutes. Samples were blocked in 5% BSA, 5% normal goat serum, and 0.1% Triton X-100 in PBS solution for 3 hours at room temperature. Primary antibodies incubated overnight in block overnight at 4°C. After four, 30-minute washes at room temperature, samples incubated in secondaries overnight at 4°C. Samples were washed four times for 30 minutes, incubated in DAPI (1:1000 in 0.1% PBST) for 15 minutes, washed and stored in mounting media (90% glycerol and 20% n-propyl gallate) at 4°C until slide preparation.

For EdU incorporation- Ovaries were dissected, and incubated in EdU solution for 1 hour, then followed typical fixation and staining protocol.

For pMad antibody staining- ovaries were dissected in phosphatase inhibitor in PBS. Samples were fixed in 4% PFA in PBS for 15 minutes. Samples were rinsed with 0.3% PBST and washed

3 times for 10 minutes. Samples incubated in primary antibody in block (3% BSA in 0.3% PBST) overnight at 4 degrees C. Samples then followed typical fixation and staining protocol. *For VASA staining*- ovaries were dissected, fixed, and stained as described in Greider et al., 2000.

Microscopy

Mounted ovaries were imaged with the Zeiss LSM700 confocal microscope using 1-micron optical sections using Zen Black.

Phenotypic analysis

GSCs were identified by fusome morphology adjacent to the cap cells according to Ables & Drummond-Barbosa, 2013. Germaria were counted as tumorous by high number of cystoblast-like cells and an absence of a 16-cell cyst. Egg chambers were identified if there were enlarged germ cell nuclei encased by a monolayer of follicle cells. Cyst collisions were determined by lack of follicle cells partitioning adjacent cysts in region 2B/3 of the germarium. To capture aspect ratio of 16-cell cysts, the 'measure' tool in Zen Blue was used to measure the X and Y-axis at the z-stack with most central view of the cyst. The 'measure' tool was also used to measure distance between cysts at the z-stack with closest proximity.

Statistical analysis

Student's T-test (two tailed, unequal variance) was performed in Excel. Fisher's exact for was performed in Graph Pad Prism.

Ovary dissection and dissociation for single-cell RNA-sequencing

Protocol was adapted from Method A in Slaidina et al., 2021. 100 ovaries from each genotype were dissected in Grace's insect medium and disassociated in a 0.5% type 1 collagenase, 1% trypsin 1:250 in PBS solution for 15 minutes using vigorous pipetting every few minutes to enhance dissociation. Disassociation was stopped by adding Grace's insect medium with 10% fetal bovine serum. Suspended cells were strained through a Flowmi 40-micron filter tip and then centrifuged at 5 rpm for 3 minutes. Supernatant was decanted, cells were resuspended in 0.04% BSA in PBS and centrifuged again at 5 rpm for 3 minutes. Final suspension was in Grace's insect medium and then preserved on ice. Live/dead count was measured on the Invitrogen Countess 3FL Cell Counter. Approximately $1.70x10^6$ cells from the TkvACT sample were collected with 89% being live. Approximately $1.17x10^7$ cells from the EcR.B1 sample were collected with 95% being live.

scRNA-seq data analysis

Library was constructed using 10X Genomics Chromium single cell 3' V3 kit. 5,741 cells were sequenced from the EcR.B1 sample with a total of 136,771,995 reads (an average of 23,799 reads per cell). 96.70% of reads were valid barcodes and 74.6% mapped confidently to the genome. The median UMI counts per cell was 4,112. For the Tkv^{ACT} sample, 7,043 cells were sequenced with a total of 116,346,480 reads (an average of 16,519 per cell). 97.40% of reads were valid barcodes and 96.8% mapped confidently to the genome. The median UMI count per cell was 6,934. The aggregated data set of EcR.B1 and Tkv^{ACT} were analyzed using the 10X Loupe Browser platform. The aggregated data of both samples pipeline-generated 16 clusters. When measuring differentially expressed genes between clusters and groups, Log2 fold change is

the ratio of the normalized mean gene UMI counts in the cluster relative to all other clusters. P-values were adjusted using the Benjamini-Hochberg correction for multiple tests.

***DROSOPHILA* STOCKS**	**Source**	**Identifier**
UASp-LacZ	D. Drummond-Barbosa	
UASp-tubGFP	Bloomington Stock Center	BDSC #7373
Nos-gal4::VP16-nos.UTR	D. Drummond-Barbosa (Rørth, 1998b; Van Doren et al., 1998)	BDSC #4937 or 7253
P{otu-GAL4::VP16.R}1, w1118	Bloomington Stock Center	#58424
y[1] w[*]; P{w[+mC]=GAL4-nos.NGT}40; P{w[+mC]=GAL4-nos.NGT}A	Bloomington Stock Center	#64308
Bam>Gal4 ,Bam>Gal4/CyO;Bam>Gal4/TM6	(Clemot et al., 2018)	
FlyFos015150(pRedFlp-Hgr)(bam29830::2XTY1-SGFP-V5-preTEV-BLRP-3XFLAG)dFRT	Vienna Drosophila Resource Center	318001
Dad-lacZ	D. Drummond-Barbosa and T. Xie (Kai and Spradling, 2003; Song et al., 2004)	
y[1] w[*]; TI{GFP[3xP3.cLa]=CRIMIC.TG4.1}aub[CR02423-TG4.1]/SM6a	Bloomington Stock Center	92663
UASz-EcR.B1 ΔC655/CyO	This paper	
UASz-EcR.B1 F656A/CyO	This paper	
UASz-EcR.B1 W650A/CyO	This paper	
UASz-EcR.B1 DBL/CyO	This paper	
UASz-mCherry::EcR.B1/CyO	This paper	
UASz-EcR.B1::mCherry/CyO	This paper	
UASz-EcR.A ΔC627/CyO	This paper	
UASz-EcR.A F617A/CyO	This paper	
UASz-EcR.A W622A/CyO	This paper	
UASz-EcR.A DBL/CyO	This paper	
UASz-mCherry::EcR.A/CyO	This paper	
UASz-EcR.::mCherry/CyO	This paper	
UASp-EcR RNAi 6-4	This paper	
CHEMICALS AND REAGENTS	**Source**	**Identifier**
Grace's insect medium	Caisson Labs	

Phosphate buffered saline (pH 7.4)	Fisher Scientific	
Triton X-100	Fisher Scientific	BP151-500
5% bovine serum albumin	Sigma Aldrich	
5% normal goat serum	MP Biomedicals	
16% Paraformaldehyde	Fisher Scientific	18505
Phosphatase Inhibitor Mini Tablets	Thermo Fisher	A32957
ANTIBODIES AND STAINS		
Mouse anti-Hts (1:10)	DSHB	1B1; RRID: AB_528070
Mouse anti-LamC (1:100)	DSHB	LC28.26; RRID: AB_528339
Mouse anti-LamB (1:100)	DSHB	ADL67.10
Chicken anti-GFP (1:2000)	Abcam	#AB13970
Rabbit anti-VASA (1:1000)	P. Lasko (Lasko & Ashburner et a., 1990)	
Mouse anti-orb (1:500)	DSHB	4H8
Mouse anti-orb (1:500)	DSHB	6H4
Guinea pig anti-RbFox1	M. Buszczak (Tastan et al., 2010)	
Chicken anti-β gal	Abcam	AB9361
X anti-PHH3 (1:200)	Millipore	#06-570
Click-iT EdU Cell Proliferation Kit for Imaging, Alexa Fluor 647 dye	Fisher Scientific	Invitrogen C10340
Rabbit anti-pMad (1:100)	Abcam	AB52903
Rat anti-dsRed (1:500)	Takara	632496
Goat anti-mouse AlexaFluor 488, 568, 688 (1:200)	Life Technologies	
Goat anti-chicken AlexaFluor 488 (1:200)	Life Technologies	
Goat anti-rabbit AlexaFluor 488, 568 (1:200)	Life Technologies	
Goat anti-rat Alexa Fluor 488 (1:200)	Life Technologies	
4'-6-diamidino-2-phenylindole (DAPI) (0.5 μg/mL)	Sigma	
SOFTWARE		
Excel	Microsoft	
Zen Blue	Zeiss	
Zen Black	Zeiss	
Prism 10	Graph Pad	
Loupe Browser 7	10X Genomics	
Photoshop	Adobe	
Illustrator	Adobe	

PLASMIDS		
pUASz-1.0	Drosophila Genetic Resource Center	
mCherry	R. Hughes	
pUASz-1.0-EcR.B1 ΔC655		
pUASz-1.0-EcR.B1 F645A		
pUASz-1.0-EcR.B1 W650A		
pUASz-1.0-EcR.B1 DBL		
pUASz-1.0-mCherry::EcR.B1		
pUASz-1.0-EcR.B1::mCherry		
pUASz-1.0-EcR.A ΔC627		
pUASz-1.0-EcR.A F617A		
pUASz-1.0-EcR.A W650A		
pUASz-1.0-EcR.A DBL		
pUASz-1.0-mCherry::EcR.A		
pUASz-1.0-EcR.A::mCherry		
pUC57-EcR.B1 ΔC655		
pUC57-EcR.B1 F645A		
pUC57-EcR.B1 W650A		
pUC57-EcR.B1 DBL		
pUC57-EcR.A ΔC627		
MOLECULAR CLONING REAGENTS		
PrimeSTAR HS premix	Takara	#R040A
BamH1-HF	New England BioLabs	
In-fusion Snap Assembly Cloning	Takara	#638948
Stellar Competent Cells		
Wizard Plus SV Minipreps DNA Purification System	Promega	A1460
Monarch PCR & DNA Cleanup Kit	New England BioLabs	#T1030
PRIMERS		
EcR.B1 deltaC655 FWD EcR.B1 F645A FWD EcR.B1 W650A FWD EcR.B1 DBL FWD		AAAAATCAAAGGA TCATGAAGCGGCG CTGGTCG
EcR.B1 deltaC655 REV EcR.B1 F645A REV		TACCCTCGAGGGA TCCTAGATGGCAT GAACGTCCCAG
EcR.B1 W650A REV EcR.B1 DBL REV		TACCCTCGAGGGA TCCTAGATGGCAT GAACGTCGGC

EcR.A deltaC627 FWD		AAAAATCAAAGGA TCATGTTGACGAC GAGTGGACAAC
EcR.A deltaC627 REV		TACCCTCGAGGGA TCCTAGATGGCAT GAACGTCCCAG
mCherry FWD (for mCherry::EcR.B1 and mCherry::EcR.A))		TCA TGC CAT CAT GGT GAG CAA GGG CGA GG
mCherry REV (for mCherry::EcR.B1 and mCherry::EcR.A)		AGT GGT ACC CTC GAG GGA TCT TAC TTG TAC AGC TCG TCC ATG CC
EcR.B1 FWD (for mCherry::EcR.B1)		AAA AAA AAA ATC AAA GGA TCA TGA AGC GGC GCT GGT CG
EcR.B1 REV (for mCherry::EcR.B1)		TGC TCA CCA TGA TGG CAT GAA CGT CCC AGA TCT CC
EcR.A FWD (For mCherry::EcR.A)		AAA AAA AAA ATC AAA GCA TCA TGT TGA CGA CGA GTG GAC AAC
EcR.A REV (for mCherry::EcR.A)		TGCTCA CCA TGA TGG CAT GAA CGT CCC AG
EcR.B1 FWD (for EcR.B1::mCherry)		AAAAAAAAAATCA AAGGATCATGAAG CGGCGCTGGTCG
EcR.B1 REV (for EcR.B1::mCherry)		TGCTCACCATGAT GGCATGAACGTCC CAGATCTCC
mCherry FWD (for EcR.B1::mCherry)		TCATGCCATCATG GTGAGCAAGGGCG AGG
mCherry REV (for EcR.B1::mCherry)		AGTGGTACCCTCG AGGGATCTTACTT GTACAGCTCGTCC ATGCC
EcR.A FWD (for EcR.A::mCherry)		AAAAAAAAAATCA AAGGATCATGTTG ACGACGAGTGGAC AAC

EcR.A REV (for EcR.A::mCherry)		TGCTCACCATGAT GGCATGAACGTCC CAG
mCherry FWD (for EcR.A::mCherry)		TCATGCCATCATG GTGAGCAAGGGCG AGG
mCherry REV (for EcR.A::mCherry)		AGTGGTACCCTCG AGGGATCTTACTT GTACAGCTCGTCC ATGCC
EcR.A F617A FWD		AGGCCCTCGAGGA GATCTGGGACGTT CATGCCATC
EcR.A F617A REV		TCTCCTCGAGGGC CTTGGGCAGTTTG CGGTTTTTG
EcR.A W622A FWD		GGAGATCGCCGAC GTTCATGCCATCT AGGATCCC
EcR.A W622A REV		ACGTCGGCGATCT CCTCGAGGAACTT GGGC
EcR.A DBL FWD		CCCTCGAGGAGAT CGCCGACGTTCAT GCCATCTAGGATC CC
EcR.A DBL REV		CGATCTCCTCGAG GGCCTTGGGCAGT TTGCGGTTTTTG
scRNA-seq REAGENTS		
1% Collagenase	Thermo Fisher	#17018-029
0.5% Type 1 Trypsin	Thermo Fisher	#27250018
Fetal Bovine Serum	Invitrogen	#10082-147
Flowmi Tip Strainers	Sigma Aldrich	H13680-0040
10X Chromium single cell 3' reagent kit V3	10X Genomics	

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