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1 Steroid signaling controls sex-specific development in an invertebrate

2

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14 **Summary**

15 In vertebrate sexual development, two important steroid hormones, testosterone and estrogen,
16 regulate the sex-specific development of many tissues. In contrast, invertebrates utilize a single
17 steroid hormone, ecdysone, to regulate developmental timing in both sexes. However, here we
18 show that in *Drosophila melanogaster*, sex-specific ecdysone (E) signaling controls important
19 aspects of gonad sexual dimorphism. Rather than being regulated at the level of hormone pro-
20 duction, hormone activity is regulated cell-autonomously through sex-specific hormone recep-
21 tion. Ecdysone receptor (EcR) expression is restricted to the developing ovary and is repressed in
22 the testis at a time when ecdysone initiates ovary morphogenesis. Interestingly, EcR expression
23 is regulated downstream of the sex determination factor Doublesex (Dsx), the founding member
24 of the Dsx/Mab3 Related Transcription Factor (DMRT) family that regulates gonad development
25 in all animals. E signaling is required for normal ovary development[1,2], and ectopic activation of
26 E signaling in the testis antagonized stem cell niche identity and feminized somatic support cells,
27 which were transformed into follicle-like cells. This work demonstrates that invertebrates can
28 also use steroid hormone signaling to control sex-specific development. Further, it may help ex-
29 plain recent work showing that vertebrate sexual development is surprisingly cell-autonomous.
30 For example, chickens utilize testosterone and estrogen to control sex-specific development, but
31 when they have a mixture of cells with male and female genotypes, the male cells develop as
32 male and the female cells develop as female despite exposure to the same circulating hormones[3].
33 Sex-specific regulation of steroid hormone signaling may well underly such cell-autonomous
34 sexual fate choices in vertebrates as it does in *Drosophila*.

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35 Sexual dimorphism is a hallmark feature of metazoans and encompasses all discernible dif-
36 ferences between males and females of the same species. Sex-specific gonad development is in-
37 structed by members of the Doublesex/Mab-3 Related Transcription Factor (DMRT) family in
38 all animals for which their role has been characterized, including flies, nematodes, chickens,
39 mice, and humans[4]. *DMRT1*-mutant male mice initiate testis development but the gonad then be-
40 comes feminized[5,6], whereas in rabbits, loss of DMRT1 causes earlier sex reversal with ovary
41 formation in XY embryos[7]. Humans that are XY, but also heterozygous for mutations in
42 DMRT1, exhibit a range of phenotypes, including complete gonadal dysgenesis with sex rever-
43 sal. Interestingly, these patients also have persistent Mullerian Ducts indicating an early problem
44 in testis development (defects in Sertoli cells that produce anti-Mullerian Hormone)[8].

45 The DMRT family was founded by the discovery of *doublesex (dsx)* in flies[9]. *Drosophila* sex
46 determination leads to alternative splicing of *dsx* RNA and the production of either male or fe-
47 male isoforms of Dsx protein (Dsx^{M} or Dsx^{F}), which share the same DNA binding domain but
48 have opposite effects on target gene transcription. *dsx* controls all known aspects of sexual di-
49 morphism in the somatic gonad, including important components of the germline stem cell niche
50 (the "hub" in males and terminal filaments (TFs) and cap cells in females), and the somatic cells
51 that support gametogenesis (cyst stem cells and cyst cells in males, and escort cells, follicle stem
52 cells and follicle cells in females)(reviewed by[10]). The timing of gonad development is also sex-
53 ually dimorphic, with the testis being formed by the end of embryogenesis (24 hrs after fertiliza-
54 tion, AF), while ovary development occurs several days later in the mid-third larval instar (L3)
55 stage (4 days AF).

56 In mammals, the steroid hormones testosterone and estrogen control many aspects of sex-
57 specific development in a variety of tissues. In contrast, invertebrates like *Drosophila* utilize the
58 same steroid hormone, ecdysone (E), in both sexes to regulate developmental timing and adult
59 female reproduction[11]. The ecdysone receptor (EcR) is a nuclear hormone receptor, similar to the
60 mammalian testosterone and estrogen receptors, that is activated by 20-hydroxyecdysone (20E).
61 EcR-dependent transcriptional activation requires a number of co-factors that are also conserved
62 in mammals, including the RXR ortholog Ultraspiracle (Usp) and the SRC-3 homolog Taiman
63 (Tai). EcR target gene activation has been implicated in the establishment of the female gonad
64 niche during development[1,2] although its role in regulating sex-specific gonadogenesis has not
65 been examined.

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66

67 ### *dsx* regulates ecdysone signaling in the gonad

68 We examined transcriptional activation via EcR in developing gonads using a transgenic
69 reporter containing EcR binding sites (*EcRE-lacZ*)[12,13] and immunostaining for the EcR target
70 Broad (Br)[2]. In late larval ovaries, *EcRE-lacZ* produced high levels of β-gal throughout the apical
71 cap, including TF cells (Fig. 1a). Similarly, Br was expressed strongly in all somatic cells of the
72 late larval ovary, including the developing TFs[2] (Extended Data Fig. 1a). In the larval testis,
73 however, little to no *EcRE-lacZ* activity or Br expression was detected, including in the testis
74 niche (Fig. 1b outline, Extended Data Fig. 1b). However, Br was detected in the testis terminal
75 epithelium cells (Extended Data Fig. 1c), indicating that the testis environment receives 20E and
76 supports ecdysone signaling in the cells that can respond. The Z1 isoform of Br is the most
77 ecdysone-responsive isoform in the developing ovary[11] (Extended Data Fig. 1d), and Br-Z1 was
78 consistently absent from the male stem cell niche (Extended Data Fig. 1e).

79 In mammals, sex-biased hormone activity is regulated at the level of hormone produc-
80 tion: females produce higher levels of estrogen, while males produce higher levels of testos-
81 terone. In contrast, we found that a female bias in hormone levels could not account for the ob-
82 served dimorphism in ecdysone activity. Supplying exogenous 20E, or a membrane-permeable
83 EcR agonist (chromafenozide), did not activate E signaling in testes but was able to induce
84 premature EcR transcriptional activity in younger L3 ovaries (Extended Data Fig. 2a-f).
85 Ecdysone is converted to its active form, 20E, by the P450 enzyme Shade[27] (Shd). We found that
86 depletion of *shd* from the ovary did not reproduce female niche defects that are seen upon block-
87 ing EcR activity[2] (Extended Data Fig. 2g-h). We conclude that the female bias in E signaling is
88 not due to sex-specific production or availability of 20E.

89 We previously conducted a collaborative genome-wide search for putative Dsx targets
90 using genomic and computational approaches[14]. We identified numerous sequences across the
91 *EcR* locus with high similarity to the Dsx consensus binding sequence, many of which were con-
92 served in other *Drosophila* species and could be bound by Dsx based on DamID-seq[14]. We there-
93 fore explored whether sex differences in *EcR* expression might account for sexually dimorphic E
94 signaling in the gonad. EcR was expressed at high levels in the female somatic gonad (Fig. 1c)
95 and at very low levels in the larval testis (Fig. 1d). We also saw expression of EcR in the termi-
96 nal epithelium of the testis (Extended Data Fig. 1h, k) which were the only cells of the testis that

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97 expressed the *EcR* target BrC (Extended Data Fig. 1c). An EcR co-factor, Taiman (Tai), that me-
98 diates ecdysone signaling in the adult ovary[15] was expressed in the somatic gonad of both larval
99 ovaries and testes (Fig. 1e-f).

100 To test whether EcR expression and activity are regulated downstream of *dsx*, we as-
101 sessed EcR and Br levels in *dsx*-mutant gonads. We generated female animals heterozygous for
102 the dsx^{D} allele[16], which cannot be spliced into the dsx^{F} isoform. Thus, XX $dsx^{D}/+$ animals ex-
103 press both Dsx^{M} and Dsx^{F}, which antagonize one another[16]. In the gonad, this leads to stochastic
104 establishment of male or female niche structures (hubs or TFs)[17]. Importantly, all other aspects of
105 the female sex determination pathway besides *dsx* are present in these animals, making this a
106 good test of *dsx* function. We found that XX $dsx^{D}/+$ gonads exhibited lower levels of EcR pro-
107 tein (Fig. 2a-d) and *EcR* mRNA (Extended Data Fig. 3a-c) compared with control ovaries. While
108 Br-Z1 was predictably female-specific in control gonads (Fig. 2e-f), in $dsx^{D}/+$ gonads Br-Z1 ex-
109 pression in the niche correlated with niche identity; $dsx^{D}/+$ gonads with a hub expressed very
110 low levels of Br-Z1 (Fig. 2g) while $dsx^{D}/+$ gonads with TFs expressed higher levels of Br-Z1
111 (Fig. 2h), supporting the idea that high ecdysone signaling promotes female niche development.

112 To evaluate whether *dsx* is a transcriptional regulator of *EcR*, we generated a transgenic
113 reporter, EcR^{6kb}-*gfp*, that expresses GFP under control of a 6.3-kb intronic enhancer element in
114 the *EcR* locus and is expressed in the somatic gonad. EcR^{6kb}-*gfp* was expressed in somatic cells
115 of the larval ovary, while very low levels were observed in the larval testis (Fig. 2i, j). Interest-
116 ingly, EcR^{6kb}-*gfp* activity was lower in XX $dsx^{D}/+$ gonads compared with control XX gonads
117 (Fig. 2k-m; Extended Data Fig. 3d-f) indicating that the effect of *dsx* on *EcR* expression is tran-
118 scriptional.

119

120 **EcR antagonizes testis development**

121 Since EcR activity was female-biased during gonad development, we investigated wheth-
122 er the requirement for *EcR* in gonad niche development is sexually dimorphic. EcR activation by
123 20E is required for development of the female gonad stem cell niche[1,2]. To investigate the role of
124 EcR in gonad niche development, we blocked EcR activation in the somatic gonad by over-
125 expressing a dominant-negative form of *EcR-A*[18] (EcR^{DN}) using the somatic gonad driver *traffic*
126 *jam (tj)-GAL4* (*tj-GAL4; UAS-*EcR^{DN}, abbreviated as *tj*>EcR^{DN}). EcR acts as a repressor in the
127 absence of 20E[19], which is required to prevent premature ovary development[2], preventing the use

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128 of *EcR* null alleles for this experiment. However, expression of EcR^{DN} specifically blocks the
129 activator function of EcR[18]. As previously observed[2], we found that EcR^{DN} expression in the so-
130 matic gonad prevented the differentiation of female niche structures (TFs, Fig. 3a, b). In contrast,
131 transcriptional activation via EcR was not required for establishment of the male gonad niche, as
132 testes expressing EcR^{DN} did not contain fewer hub cells than control testes. In fact, *tj*>EcR^{DN} tes-
133 tes showed a slight increase in hub cell number and hub volume compared with controls (Ex-
134 tended Data Fig. 4).

135 To test whether activating EcR in the developing testis is deleterious to male niche devel-
136 opment, we first identified the rate-limiting components for E signaling in the larval testis. Over-
137 expression of EcR alone was not sufficient to activate signaling, as determined by quantifying Br
138 expression (Extended Data Fig. 5a-e). However, co-expression *EcR* along with its co-factor
139 *taiman* (*tj>EcR,tai*) in the male somatic gonad led to a significant increase in Br expression (Ex-
140 tended Data Fig. 5a-e) indicating we could now ectopically activate E signaling in the testis. This
141 led to a significant decrease in larval hub cell number compared with control testes (Fig. 3c-e)
142 which was apparent as early as the L1 stage (24-48 hrs AF, Fig. 3e and Extended Data Fig. 6a-c).
143 Hub cell loss continued beyond larval development, as some *tj>EcR,tai* pupal testes lacked hubs
144 and, consistent with this, also lost the germline and somatic support cells dependent on the male
145 niche (Extended Data Fig. 6d-e). While EcR activation caused a loss of male niche identity, we
146 did not find a transformation to female niche identity, as occurs in loss of *dsx* function[17]. Expres-
147 sion of the female TF marker Engrailed increased in male somatic cells upon activation of E sig-
148 naling (Extended Data Fig. 7) but no evidence of TF formation was observed.
149

150 **EcR activity feminizes the adult testis**

151 While activation of E signaling was not sufficient to sex-transform the gonad niche, we
152 observed a striking male-to-female transformation of somatic support cells in the testis upon co-
153 expression of EcR and Tai. Support cells in the adult testis include the cyst cells that surround
154 each developing spermatogenic cyst and the cyst stem cells that produce them. In the ovary, so-
155 matic support cells include escort cells that nurture germ cells during the earliest stages of differ-
156 entiation and epithelial follicle cells, made by follicle stem cells, that support maturing oocytes.
157 The transcription factor Chronologically inappropriate morphogenesis (Chinmo) is normally ex-
158 pressed in hub cells and early cyst cells of L3 and adult testes, while in females it is expressed in

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159 germ cells but is absent from the somatic gonad at both stages[20-22] (Fig. 4a, b and Extended Data
160 Fig. 8a-b). Co-expression of EcR and Tai caused a loss of Chinmo expression in cyst cells but
161 not hub cells (Fig. 4c). Interestingly, this was accompanied by a gain in expression of the follicle
162 cell markers Fas3 and Castor (Fig. 4d-f), which also occurs upon Chinmo depletion from the tes-
163 tis[21,22] (Fig. 4g). Repression of *chinmo* and activation of *br* by ecdysone signaling has also been
164 observed in developing neuroblasts and in larval wing imaginal discs[23,24] and has been proposed
165 to regulate many aspects of development[25]. To investigate further the mutually repressive rela-
166 tionship between EcR and *chinmo* in the somatic gonad, we examined whether transcriptional
167 activation via EcR was required for the male-to-female transformation observed upon loss of
168 *chinmo*. Indeed, interfering with EcR activation by expressing EcR^{DN} blocked the follicle cell-
169 like transformation observed with loss of *chinmo* (Fig. 4g-h). Follicle-like cells were present in
170 92.3% of $tj{>}chinmo^{RNAi}$ testes, but they appeared in only 7.3% of $tj{>}EcR^{DN}; {>}chinmo^{RNAi}$ testes
171 (Fig. 4i). In addition, depletion of *chinmo* in the testis led to induction of the EcR target Br in the
172 transformed follicle-like cells (Fig. 4j-l). These data are consistent with a model where EcR/Br
173 and *chinmo* exhibit mutually repressive interactions in the somatic gonad.
174

175 **Discussion**

176 Taken together, our data demonstrate that E signaling can be used to control sex-specific
177 development in an invertebrate. This does not require sex-specific E production and, indeed, suf-
178 ficient E is produced in both sexes to activate signaling in cells competent to respond to the hor-
179 mone, such as the testis terminal epithelium (Extended Data Fig. 1a, c). We demonstrate that at
180 the time that E signaling activates ovary development, E signaling is repressed in most somatic
181 cells of the larval testis, including the hub and somatic support cells. Further, our work shows
182 that this repression is necessary for proper testis development: activation of E signaling causes a
183 loss of male niche (hub) identity and a transformation of adult somatic support cells toward a
184 female follicle cell-like identity. We also show that repression of E signaling in the testis is due,
185 in part, to *dsx*-dependent regulation of *EcR* expression. Therefore, invertebrates like *Drosophila*
186 that utilize the steroid hormone E to regulate development in both sexes can also employ this
187 same hormone to regulate sexual development via sex-specific regulation of its receptor.

188 We show that somatic gonad development and maintenance feature mutually exclusive
189 expression of the transcription factors Chinmo and Br. Repression of *chinmo* by EcR and Br has

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190 been observed in other developmental contexts[23-25], presenting this as a common regulatory
191 mechanism. Thus, sex-specific EcR expression may well be used to control sexual dimorphism
192 in other tissues such as the nervous system. It is notable that upon EcR hyperactivation in the
193 adult testis, somatic cells appear transformed to a female follicle cell-like identity, whereas hub
194 cells are lost but do not undergo a similar transformation toward a female niche identity. Male to
195 female niche transformation is observed in *dsx* mutants[17], indicating that there must be additional
196 regulatory mechanisms downstream of *dsx*, in addition to EcR, that promote sex-specific niche
197 development and maintenance. Candidates for this include *fruitless,* which encodes a transcrip-
198 tion factor involved in hub maintenance during larval stages[26], and *bric a brac 1* and *2 (bab1/2)*,
199 which are important for TF cell specification[27,28]. Interestingly, Fruitless, Bab1/2, Br, and
200 Chinmo all encode transcription factors with BTB (Broad, Tramtrack, Bric a brac) domains that
201 promote homotypic and heterotypic protein interactions. Further, they are all either predicted or
202 known Dsx target genes[1417,26,29]. Thus, the regulation of these factors by Dsx and EcR, in addi-
203 tion to their potential physical associations, could allow for a particularly rich network of regula-
204 tory interactions controlling sexual dimorphism in the gonad and other tissues.

205 The autonomous regulation of ecdysone signaling we report in *Drosophila* gonads may
206 provide a model for how cell-autonomous sexual identity is regulated in vertebrates. Birds, like
207 mammals, utilize estrogen and testosterone to control development of sexually dimorphic charac-
208 teristics. Indeed, manipulating the activity of aromatase, which converts testosterone to estrogen,
209 can induce sex-reversal during chicken development[30]. However, in birds with a mixture of cells
210 with male and female sex chromosome genotypes, the cells with a male genotype develop as
211 male and the cells with a female genotype develop as female, despite the fact that these animals
212 have a single circulating level of estrogen and testosterone[3,31]. Further, male chickens heterozy-
213 gous for a non-functional allele of *DMRT1* develop an ovary rather than testes, but all other tis-
214 sues analyzed are phenotypically male[32]. These data clearly indicate that even when estrogen and
215 testosterone are used to control sex-specific development, the responding tissues can cell-
216 autonomously differ in how they respond to these hormones. The simplest explanation for this is
217 that these tissues autonomously regulate their response to these steroid hormones according to
218 their sex, similarly to how *Drosophila* cells regulate sex-specific response to ecdysone signaling
219 in the gonads.

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Acknowledgments

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Author contributions

Conceptualization: L.G., E.J. and M.V.D.; Methodology: L.G., E.J., E.B., and M.V.D.; Investigation: L.G., E.J., and E.B.; Formal analysis: L.G. and E.J.; Validation: L.G., E.J. ad E.B.; Resources: M.V.D.; Writing – original draft: L.G.; Writing – review and editing: L.G. and M.V.D.; Supervision: L.G. and M.V.D.

Methods

Fly stocks and husbandry

The following flies were used and are described in FlyBase: *Oregon*^{R}, *dsx*^{1}, *Df(3R)dsx*^{3}, *dsx*^{D}, *msl3-gfp*, *tj-gal4 (P{GawB}NP1624)*, *EcRE-lacZ, UAS-lacZ*, *UAS-EcR.C*, *UAS-Tai*, *UAS-EcR-A*^{W650A} (referred to as *UAS-EcR*^{DN} in text), *UAS-chinmo*^{RNAi} (HMS00037) tai-GFP. *EcR*^{6kb}*-gfp* enhancer line was generated in this study; see below for cloning details.

Antibodies

The following antibodies were used: rabbit anti-Vasa (1:10,000; gift of Ruth Lehmann, MIT/Whitehead Institute, Cambridge, MA, USA), guinea pig anti-Tj (1:1000), mouse anti-Fas3 (1:100; DSHB), rat anti-N-cadherin (1:20; DSHB), mouse anti-EcR (Ag10.2; 1:20; DSHB), mouse anti-EcR-A (15G1a; 1:20; DSHB), mouse anti-EcR-B1 (AD4.4; 1:20, DSHB), mouse anti-Broad-core (25E9.D7; 1:40; DSHB), mouse anti-Broad-Z1 (1:50; DSHB), mouse anti-β-galactosidase (40-1a; 1:40; DSHB), mouse anti-Engrailed (1:10; DSHB), rat anti-Chinmo (1:5,000; gift of N. Sokol, formerly Indiana University, Bloomington, IN), rabbit anti-Castor (gift of W. Odenwald, NIH, Bethesda, MD). Cross-adsorbed secondary antisera (Invitrogen) were raised in goat and diluted to 4 μg/mL for staining.

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252 *Larval hormone feeding experiments*

253 Larval 20-hydroxyecdysone (20E) and chromafenozide (CF) feeding experiments were per-
254 formed as follows. First, embryos were collected for 4 hours on apple juice agar petri dishes sup-
255 plemented with regular yeast paste. 21 hours after the start of embryo collection, larvae that
256 hatched prematurely were removed. First instar larvae that hatched over the following 4 hours
257 were then transferred to a fresh apple juice plate supplemented with regular yeast paste. Newly
258 ecdysed third instar larvae were transferred to a fresh apple juice plate supplemented with either
259 control yeast paste (dry yeast mixed with H_2O lacking hormone), yeast paste plus 20E (dry yeast
260 mixed with 1 mM 20E in H_2O), or yeast paste plus 1 mM CF (dry yeast mixed with 1 mM CF in
261 H_2O). Wandering third instar larvae were dissected 48 hours later for gonad staining and imag-
262 ing.

263

264 *Immunofluorescence*

265 Testes and ovaries were dissected in PBS and fixed in 5% formaldehyde (diluted in PBS) for 20
266 minutes at room temperature (RT). Fixed tissue was washed twice for 10 minutes each in PBTx
267 (PBS + 0.1% Triton X-100). Blocking was performed for 30 minutes or overnight in BBTx (PBS
268 + 0.1% Triton X-100 + 1% BSA). Primary antibodies were diluted in BBTx and incubated over-
269 night at 4° C. Primary antibodies were washed off twice for 10 minutes each in BBTx. Second-
270 ary antibodies were diluted in BBTx, incubated for 2-3 hours at RT in the dark, and washed off
271 in PBTx. Samples were then incubated in 1 µg/mL DAPI for 10 minutes at RT in the dark, and
272 finally washed twice in PBTx. HCR-FISH was performed according to manufacturer's protocol
273 (Molecular Instruments, Inc.) and *EcR* hybridization probes were generated commercially. Tis-
274 sue mounting medium was supplemented with DABCO anti-fade reagent (*company*) prior to
275 confocal analysis. Confocal images were captured using a Zeiss LSM 710 confocal microscope
276 using 20x, 40x, or 63x objective lens.

277

278 *ImageJ quantifications*

279 For quantification of EcR^{6kb}-*gfp* gonad expression shown in Fig. 2, total GFP intensity from a
280 representative Z-slice was quantified for each gonad, then normalized to total DAPI intensity
281 within the same area.

282

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283 *Generating EcR^{6kb}-GFP constructs*

284 Cloning was performed using a modified version of pStinger[46] that replaces P-elements with *attB*
285 site to facilitate ΦC31-mediated, site-directed genome integration. The MCS from *pStinger*
286 (Barolo et al. 2000) was excised and ligated into *pARE-GFP*^{nls} (see Chatterjee & Bohmann 2012
287 for vector map) to generate *pStinger-attB*. The *hsp70* minimal promoter sequence was removed
288 (since the 6.3kb putative enhancer contains a core promoter) to generate *pStinger-attB*. This vec-
289 tor was used as the starting point to generate *EcR*^{6kb}*-GFP*. The 6.3-kb putative enhancer region
290 was amplified from a BAC construct containing part of the *EcR* locus (*BACR08A11*) using the
291 primers listed below, then digested with SphI/AgeI and ligated into *pStinger-attB* using the
292 NEBuilder HiFi DNA cloning assembly kit (New England Biosciences, Inc.). Positive clones
293 were screened by PCR and sequence integrity was verified using Sanger sequencing. Plasmid
294 was injected into *Drosophila* embryos and stable lines were generated via BestGene, Inc.

295 Primers:

296 EcR6kb_sphI_ageI_fwd: tagtgctactgcatagca

297 EcR6kb+sph1_ageI_rev: ctatgcagccgccatata

298

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299 Figure Legends – main body

300 Figure 1. Ecdysone signaling activity is female-biased in the developing gonad.

301 (a-b) Beta-galactosidase expression (green) from a transgenic *EcRE-lacZ*[25] reporter is higher in
302 the ovarian somatic gonad (a) than in the testicular somatic gonad (b). (c-d) EcR protein expres-
303 sion is higher in the ovary (c) than in the testis (d). (e-f) Expression of Taiman-GFP from a ge-
304 nomic duplication is detected in several somatic cell types of an L3 ovary (e), including the api-
305 cal cap, terminal filaments, and intermingled cells. In an L3 testis (d), Tai is detected at low lev-
306 els in the hub and CySCs and at higher levels in differentiating cyst cells. Vasa (red) labels the
307 germline, Traffic jam (Tj, blue) labels somatic cells. Scale bars = 25 μm. Yellow brackets indi-
308 cate TF cells; yellow dotted outline indicates the hub.

309

310 Figure 2. EcR activity is regulated by Dsx in the developing somatic gonad.

311 (a-c) EcR protein levels are higher in an L3 ovary (a, green) than in an L3 testis (b). EcR expres-
312 sion is diminished in XX *dsx*-mutant gonads (*dsx*^{D}/+, c-d). DAPI (blue) labels nuclei. (e-h) Br-
313 Z1, an EcR target, is expressed in TFs of a control ovary (e) and is absent from hub cells in a
314 control testis (f). Br-Z1 expression is high in an L3 XX *dsx*^{D}/+ TF structure (g) and low in a hub
315 structure (h). N-cad labels the gonad niche (TF or hub cells) (i-j) Expression of a 6.3-kb intronic
316 EcR enhancer (*EcR*^{6kb}*-gfp*) in larval gonads. Gonads shown in (i) and (j) contain two copies of
317 enhancer construct (homozygous). *EcR*^{6kb}*-gfp* is expressed throughout the somatic gonad of an
318 LL3 ovary (h, arrowheads) and is expressed at very low levels in the hub (arrowhead) and
319 CySCs of an L3 testis (j). (k-l) *EcR*^{6kb}*-gfp* expression is greatly reduced in *dsx*-mutant (*EcR*^{6kb}-
320 *gfp/+; dsx*^{D}/+) gonads (l) compared with control (*EcR*^{6kb}*-gfp/+; +*) ovaries (k). Heterozygous
321 expression of *EcR*^{6kb}*-gfp* in a control testis is shown in (m). In k-m, DAPI (blue) labels nuclei
322 and Tj (red) labels somatic cells. Endogenous GFP signal (without GFP antibody staining) is
323 shown in green. In i-m, arrowheads indicate TF cells and arrows indicate the hub. Scale bars =
324 25 μm.

325

326 Figure 3. EcR signaling is deleterious to male somatic gonad establishment.

327 (a) TFs are established in a control LL3 ovary (*tj>lacZ*). Engrailed (green) labels TF cells. (b)
328 Blocking EcR activation in the somatic gonad (*tj>EcR*^{DN}) prevents the establishment of terminal
329 filaments in an L3 ovary. (c) In a control L3 testis, Fas3 (green) labels intact hub cells. (d) Acti-

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330 vation of EcR by co-expressing EcR and Tai (*tj>EcR,tai*) in the male somatic gonad produces
331 smaller hubs with visibly fewer cells. Fas3 (green) labels hub cells. (g) Quantification of hub
332 cells per LL3 (blue dots) or L1 (yellow dots) testis in various genotypes. For statistical signifi-
333 cance here and in subsequent figures, asterisks indicate statistical significance by Student's t-test
334 as follows: *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001. Scale bars = 25 μm.
335
336 Figure 4. EcR activation in the testis causes adult somatic sex transformation.
337 (a-c) Chinmo expression in adult gonads and with EcR activation in the testis. In an adult ovary
338 (a), Chinmo protein (green) is absent from the female somatic gonad (arrowheads). Chinmo is
339 expressed in early female germ cells as previously observed[3]. (b) In an adult testis, Chinmo is
340 detectable in the hub and CySCs. (c) Chinmo expression is dramatically reduced upon EcR acti-
341 vation in the adult testis. (d-h) Expression of follicle cell markers Fas3 (red) and Castor (green)
342 in adult gonads. Adult ovaries (d) show expression of Fas3 (red) and Castor (green) in early fol-
343 licle cells, while in a control adult testis (e) Castor and Fas3 are absent from the cyst cell lineage.
344 In adult testes, as seen in previous figures, Fas3 is expressed in hub cells. EcR activation in the
345 testis (f) causes somatic feminization in the adult testis, characterized by the presence of Fas3-
346 and Castor-positive somatic aggregates. Depletion of *chinmo* in the male somatic gonad (g) also
347 causes somatic feminization as previously observed[3]. Blocking EcR activation by over-
348 expressing *EcR*^{DN} (h) suppresses somatic feminization in the absence of Chinmo. (i) Quantifica-
349 tion of feminization rescue in *tj>chinmo*^{RNAi} testes by blocking EcR activation. An abbreviated
350 description of phenotypic categories: 0=no feminization or germline defects; 1=germline defects
351 but no feminization; 2=mild feminization; 3=moderate feminization; 4=severe feminization;
352 5=acellular testes. Feminization and acellularity are indicated on the graph in blue. More com-
353 plete descriptions and visual examples of each phenotypic category can be found in Extended
354 Data Fig. 9. (j-l) Expression of Br-C (green) in the adult testis upon *chinmo* depletion. Br-C is
355 expressed in follicle cells during mid-oogenesis in the adult ovary (j). Br-C is expressed at mod-
356 erate levels in early cyst cells but is absent from differentiating cyst cells in an adult testis (k). In
357 a *tj>chinmo*^{RNAi} testis (l), Br-C is expressed in later cyst cells distal from the hub (hub and testis
358 apex are out of frame in l and l'). Scale bars = 50 μm.
359
360

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361 Figure Legends – Extended Data

362 Extended Data Figure 1. Expression of EcR and Br isoforms in the developing gonad.
363 (a-b) Expression of the EcR target Br-C in an LL3 ovary (a) and testis (b). Br-C expression in
364 terminal epithelium cells of a larval testis is shown in (c). Vasa (green) labels the germline and
365 Tj (blue) labels somatic cells. (d-e) Expression of Br-Z1 isoform in an LL3 ovary (d) and testis
366 (e). Vasa (magenta) labels the germline. (f-k) Expression of EcR-A (f-h) and EcR-B2 isoforms in
367 the LL3 ovary (f, i), testis (g, j) and testicular terminal epithelium cells (h, k). Vasa (green) labels
368 the germline and Tj labels somatic cells. Scale bars = 25 µm.
369
370 Extended Data Figure 2.
371 (a-f) Expression of *EcRE-lacZ* reporter in L3 ovaries (a-c) or L3 testes (d-f) upon feeding with
372 10% DMSO (vehicle, a and d), 20-hydroxyecdysone (20E, b and e), or chromafenozide (CF, c
373 and f). (g-h) Depletion of *shd* in the larval ovary does not visibly impair ovary development.
374 Note that *dcg-gal4* used in this experiment is expressed in both the ovary and surrounding fat
375 body. Vasa (green) labels the germline, N-cad (red) labels TF cells, and Tj (blue) labels inter-
376 mingled somatic cells. Scale bars = 25 µm.
377
378 Extended Data Figure 3.
379 (a-c) Detection of *EcR* mRNA (red) in LL3 gonads by HCR-FISH. A *dsx*-mutant (*dsx*^{D}/+) gonad
380 (c) shows lower *EcR* mRNA abundance than control ovaries (a). *EcR* mRNA in an LL3 testis is
381 shown in (b). (d-f) Detection of *GFP* mRNA (red) in LL3 gonads by HCR-FISH. *dsx*^{D}/+ gonad
382 (f) contains higher *GFP* mRNA abundance than control ovaries (d). Scale bars = 25 µm.
383
384 Extended Data Figure 4.
385 (a-b) Neither hub specification nor hub maintenance are compromised by blocking EcR activity
386 (*tj>EcR-*^{DN}). Fas3 (green) labels hub cells, Vasa (red) labels the germline, and Tj (blue) labels
387 somatic cells. (c-e) Quantification of hub cell number (c), hub volume (d), and germline stem
388 cell (GSC) number (e) upon blocking EcR activation in the somatic gonad. Scale bars = 10µm.
389
390 Extended Data Figure 5.

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391 (a-d) Br-C expression in LL3 control testes upon somatic over-expression of EcR (b), Tai (c), or
392 EcR and Tai simultaneously (d). (e) Quantification of relative Br-C intensity from a-d. Scale bars
393 = 25 μm.
394
395 Extended Data Figure 6.
396 (a-c) Representative images of early L1 (24-28h after egg lay, AEL) gonads, identified by the
397 presence of a Fas3-positive hub (green). Ovaries lack a hub (a) while testes contain a Fas3-
398 positive hub (green) by this stage (b). III staining. Co-expression of *EcR* and *tai* in the early so-
399 matic gonad (c) leads to a visibly smaller hub that contains significantly fewer hub cells than
400 control L1 testes (see Fig. 3e for quantification). Vasa (red) labels the germline, Tj (blue) labels
401 somatic gonadal precursor cells, which give rise to the somatic gonad in both sexes. (d-e) Pupal
402 testes upon EcR/Tai co-expression. In a control pupal testis (d), the germline and somatic stem
403 cells are maintained by the presence of a hub (Fas3, green) and thus differentiating germ cells
404 (Vasa, red) are observed in the testis. In some pupal testes, following EcR/Tai co-expression in
405 the somatic gonad (e), no Fas3-positive hub cells are present and the absence of Vasa and DAPI
406 staining indicates that both germline and somatic cell lineages have been lost. DAPI-positive
407 muscle sheath cells can be found at the periphery of the testis. Scale bars = 10 μm.
408
409 Extended Data Figure 7.
410 (a-c) Expression of the TF marker Engrailed (En) in LL3 gonads. En (green) labels TF cells in a
411 control ovary (a) but is not detected in the niche of a control testis (b). En is not detected in hub
412 cell nuclei upon EcR/Tai co-expression in the somatic gonad (c). En induction is observed in the
413 cyst cell lineage upon EcR activation (c). In b and c, the hub is outlined with a yellow dotted
414 line. Scale bars = 25 μm.
415
416 Extended Data Figure 8.
417 Mutually exclusive expression of Chinmo (red) and Br-Z1 (green) in somatic cells of LL3 gon-
418 ads. In an LL3 ovary (a), Chinmo is absent from somatic cells (a', inset arrowheads) while Br-Z1
419 is expressed broadly in the female somatic gonad. We note that Chinmo is expressed in female
420 larval progenitor germ cells (PGCs) (a'). In an LL3 testis (b), Chinmo is expressed in the hub,

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421 CySCs/cyst cells, and GSCs/early germ cells. Br-Z1 is not detectable in the hub or early cyst
422 cells. Vasa (blue) labels the germline. Scale bars = 25 μm.
423
424 Extended Data Figure 9.
425 Phenotypic category descriptions and representative images for rescue quantification in Figure
426 4i. (a) Category 0: no evidence of feminization (accumulation of Fas3/Castor-expressing somatic
427 aggregates), early/late spermatogonia and large-nuclei spermatocytes present. (b) Category 1: No
428 evidence of feminization, but germline defects seen (shortening of mitotic region and/or absence
429 of large-nuclei spermatocytes). (c) Category 2: mild feminization (a few cells express Fas3
430 and/or Castor, but aggregates have not yet formed). (d) Category 3: moderate feminization (folli-
431 cle-like aggregates present clearly expressing Fas3 and Castor, few germ cells are present and
432 mostly seem arrested in spermatogonial stage). (e) Category 4: severe feminization (large folli-
433 cle-like aggregates present, few/no germ cells remain). (f) Category 5: acellular. Vasa (blue) la-
434 bels the germline. Scale bars = 50 μm.
435

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Figure 1

EcRE-lacZ ovary (XX)
EcRE-lacZ testis (XY)
control ovary (XX)
control testis (XY)
Tj Vasa β-gal
β-gal
Tj Vasa EcR
EcR
control ovary (XX)
control testis (XY)
Tj Vasa Tai-GFP
Tai-GFP

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Figure 2

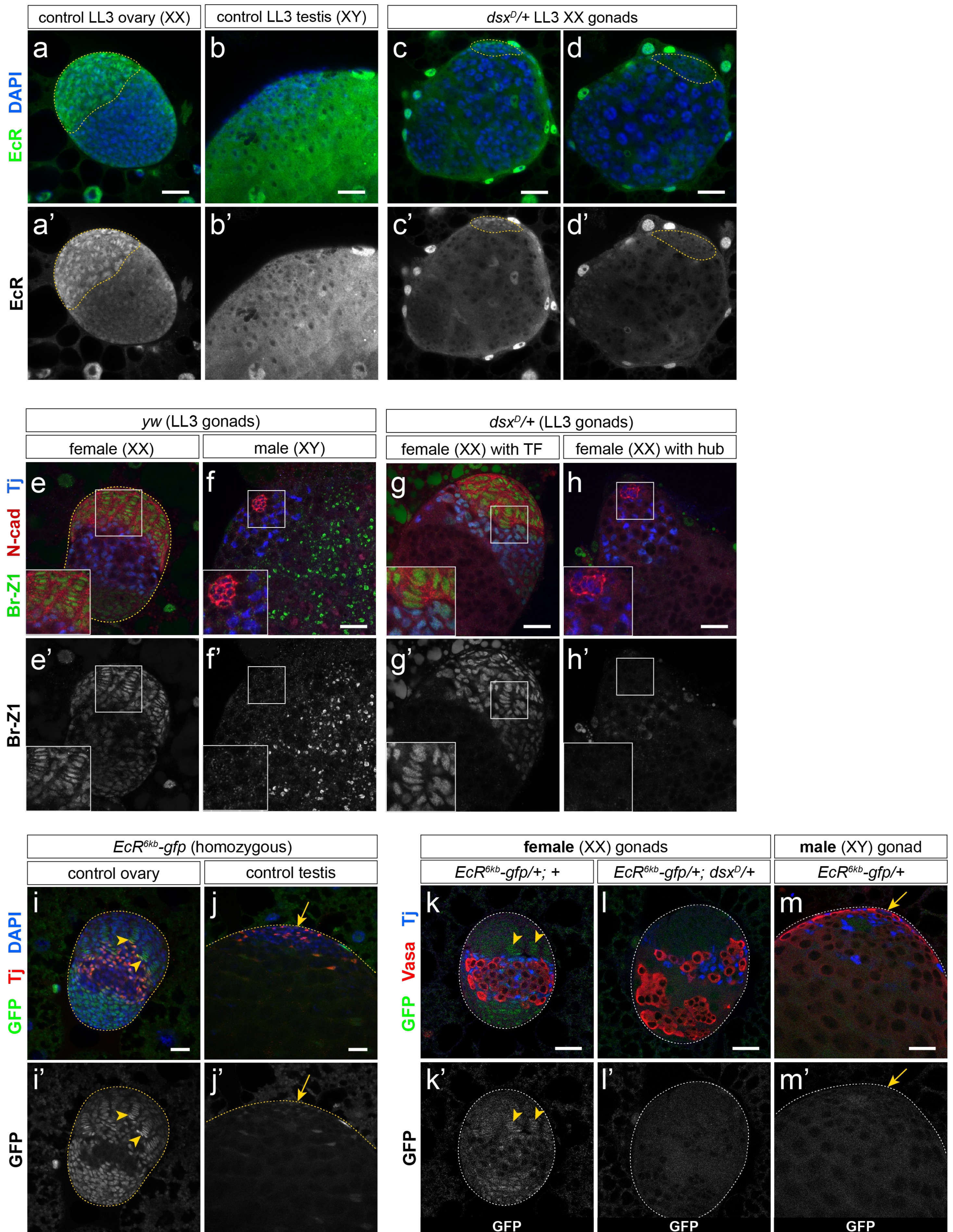

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Figure 3

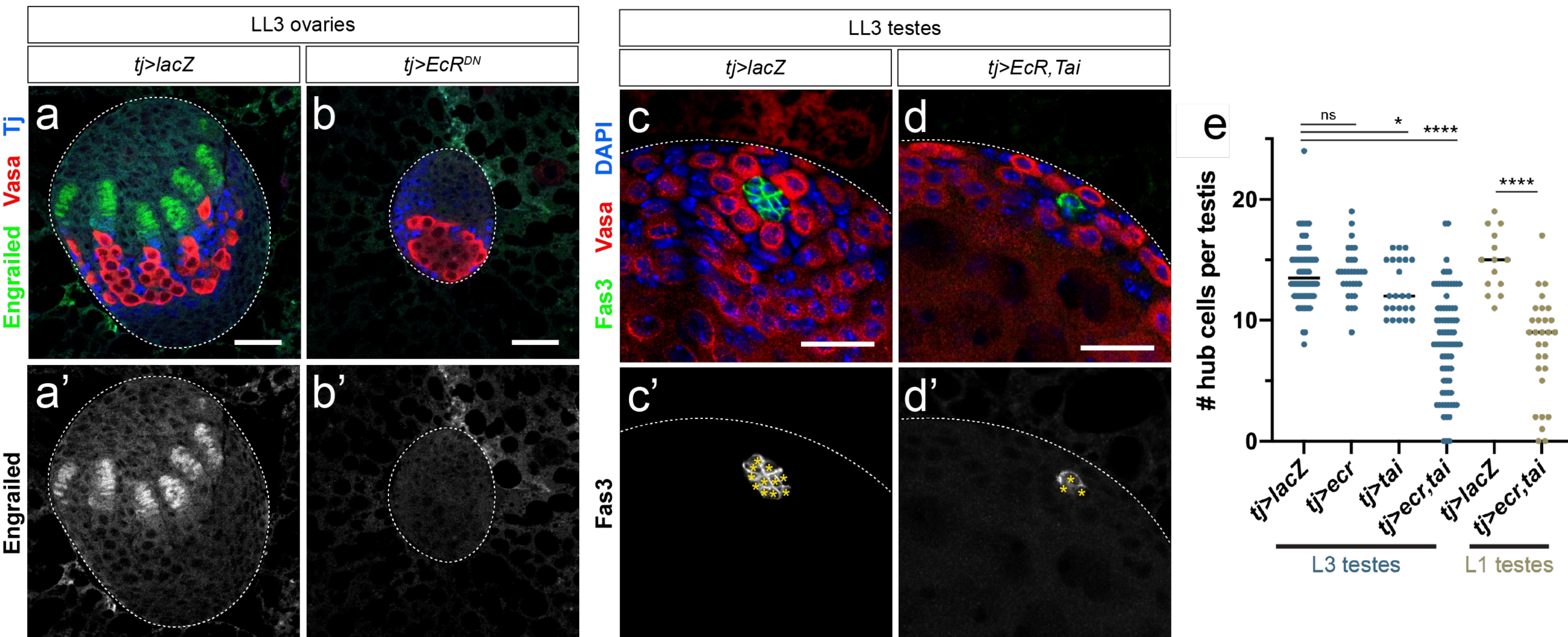

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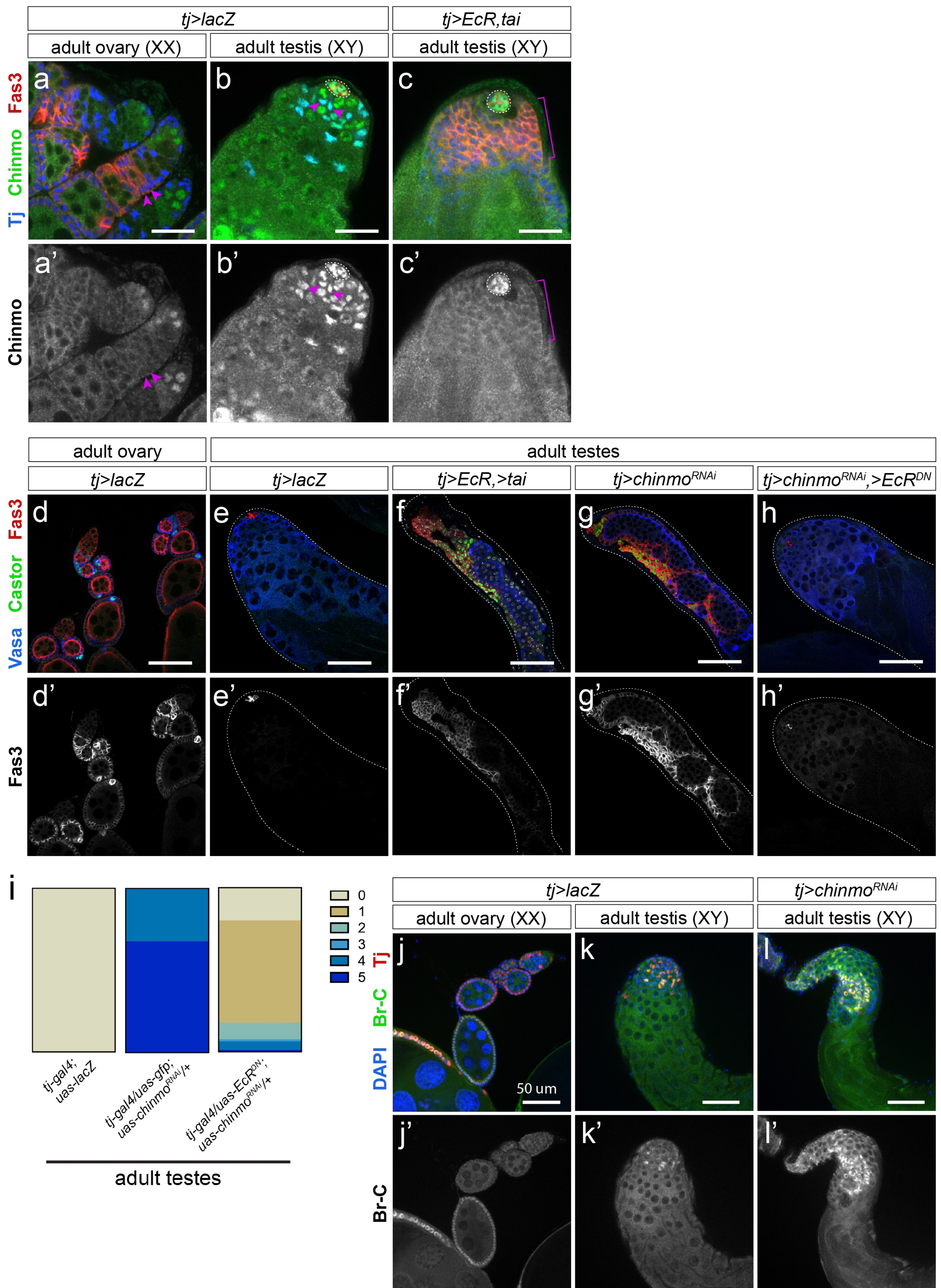
Figure 4



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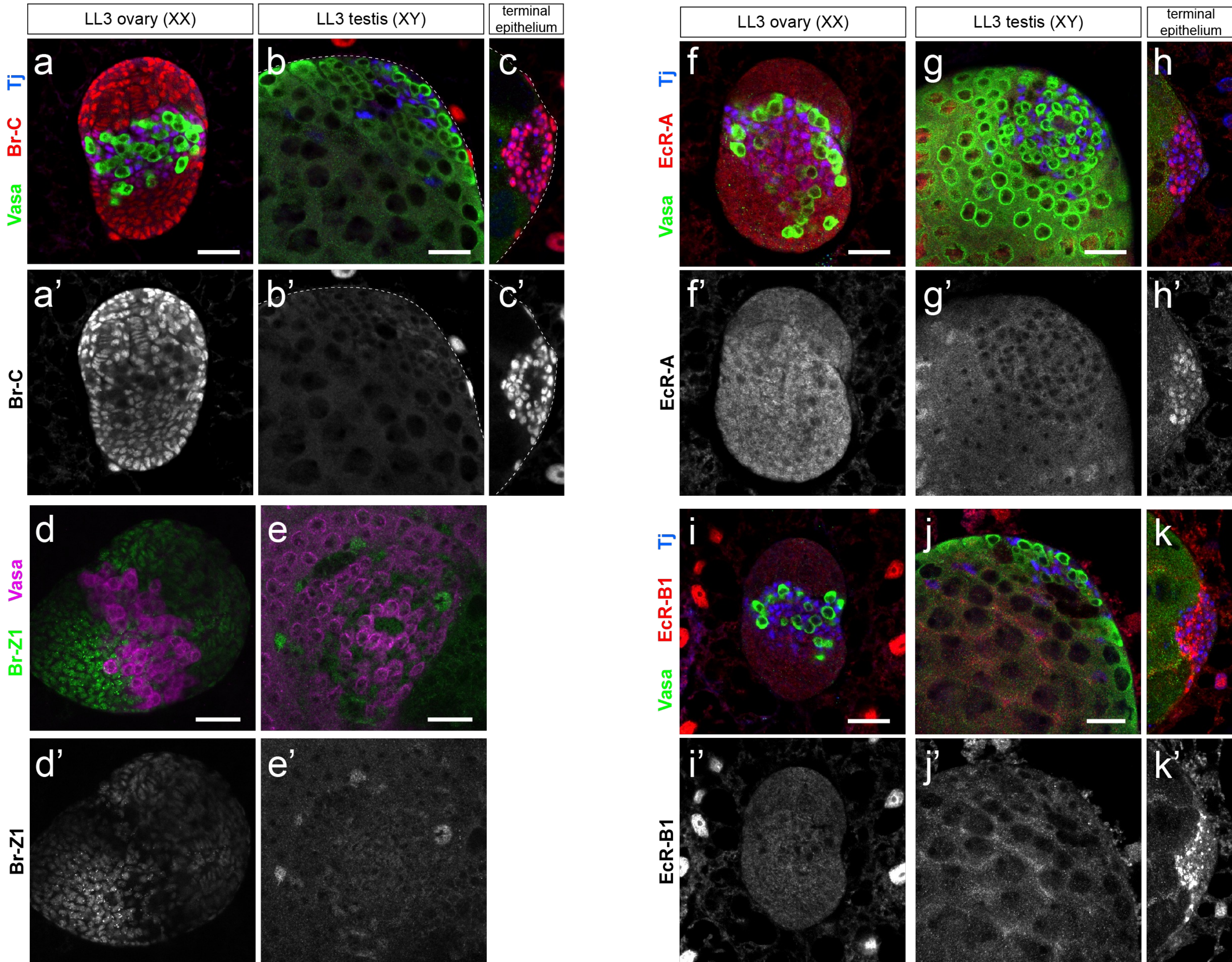
Extended Data Figure 1



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Extended Data Figure 2

EcRE-lacZ ovaries (XX)

EcRE-lacZ testes (XY)

β-gal Tj Vasa

β-gal

a a' d d'

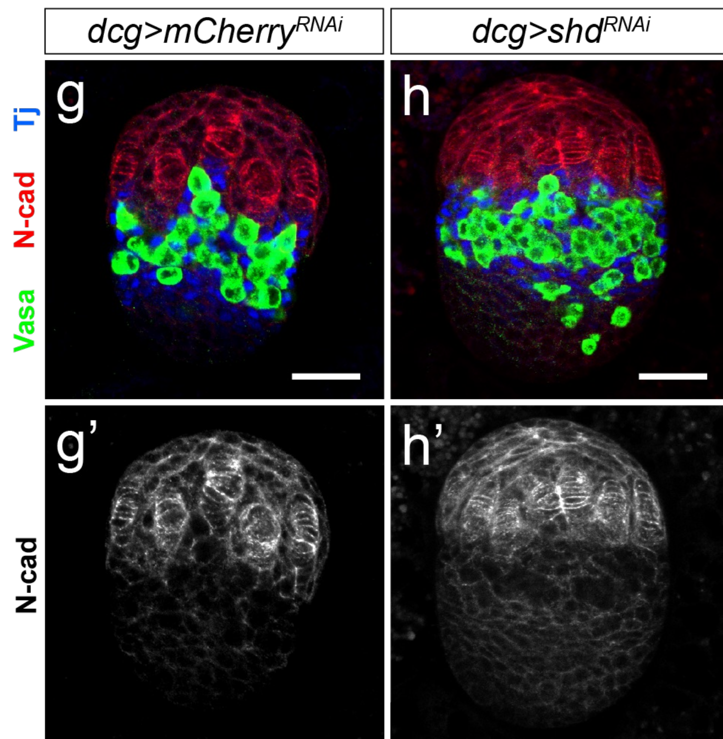
10% DMSO

b b' e e'

20E feeding

c c' f f'

CF feeding



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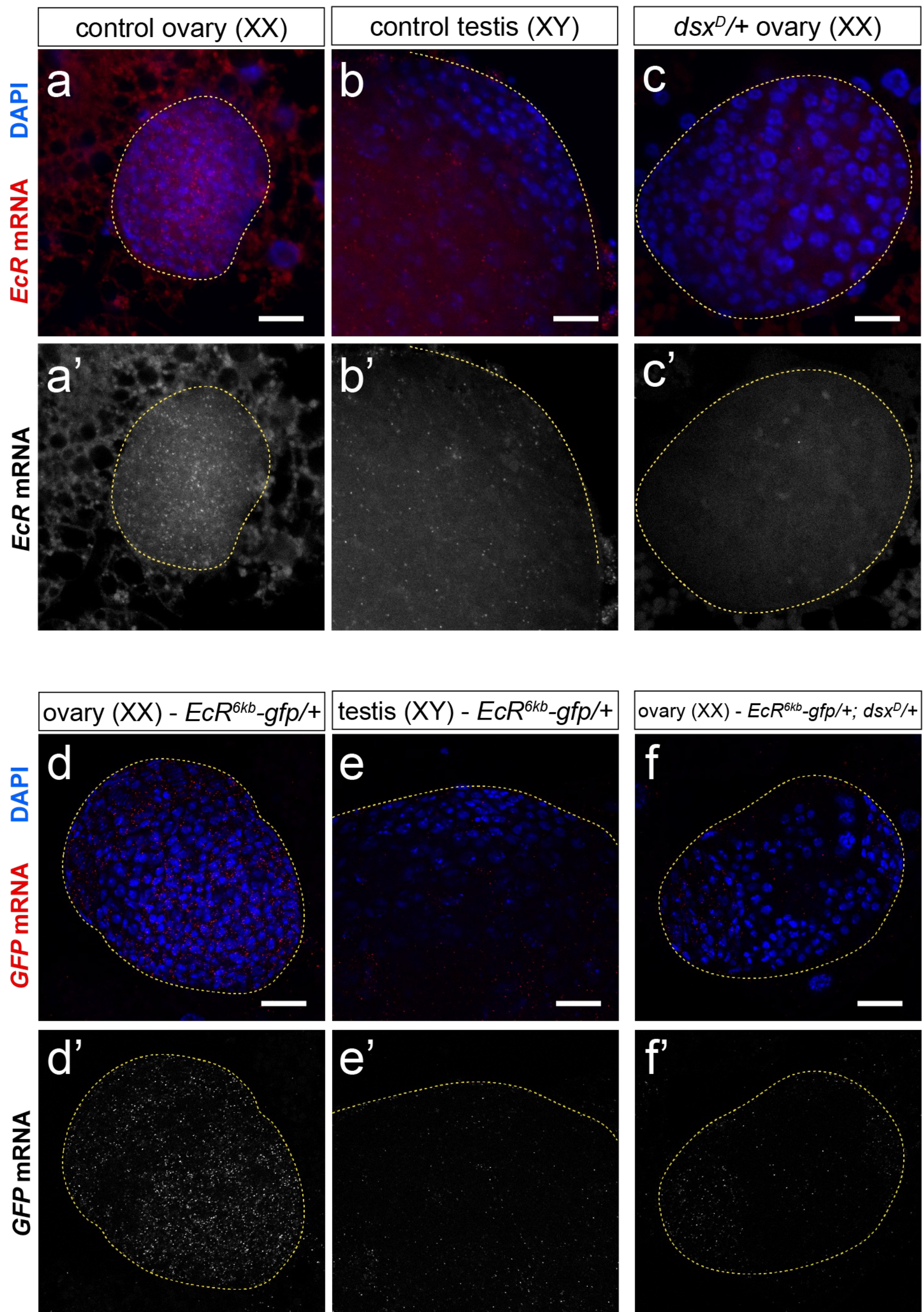
Extended Data Figure 3



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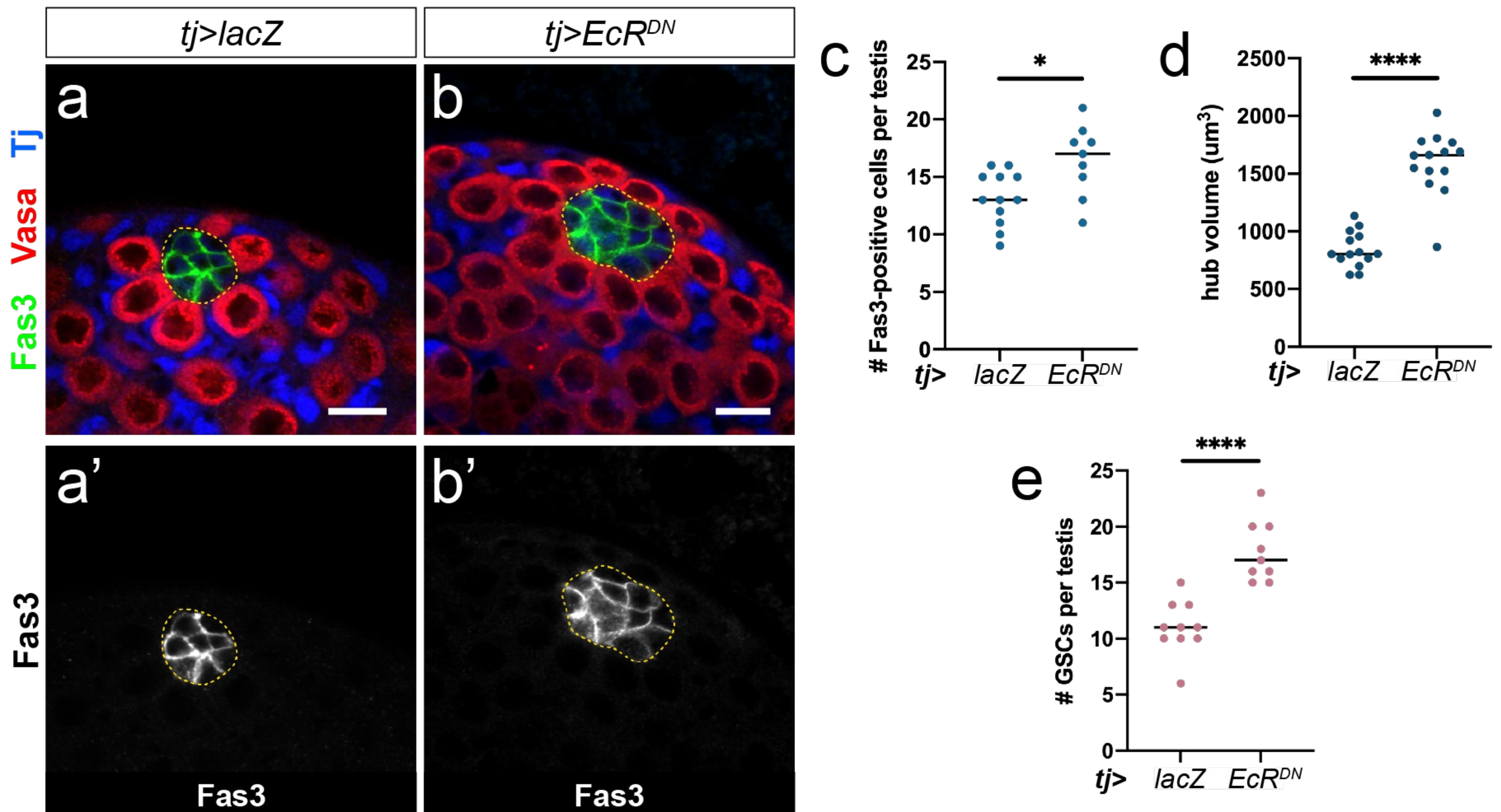
Extended Data Figure 4



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Extended Data Figure 5

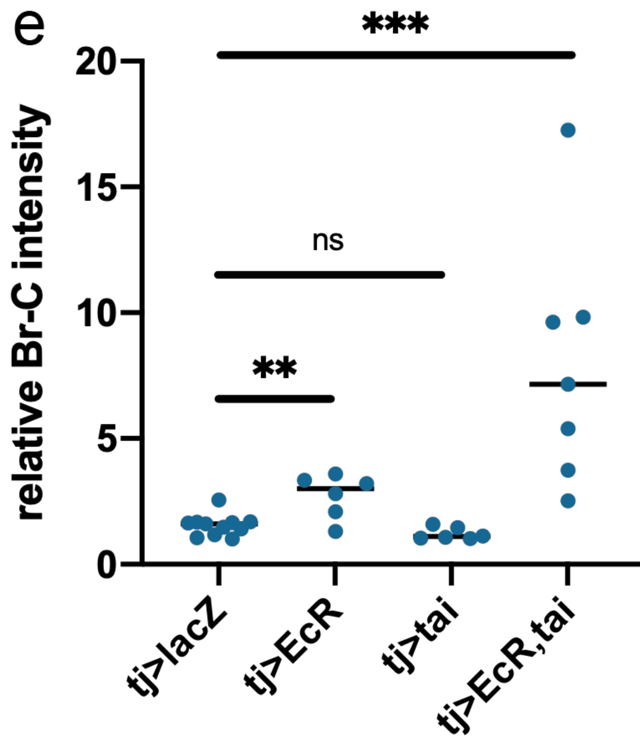
tj>lacZ | *tj>EcR* | *tj>tai* | *tj>EcR,tai*

Tj Vasa Br-C

a b c d

a’ b’ c’ d’

Br-C



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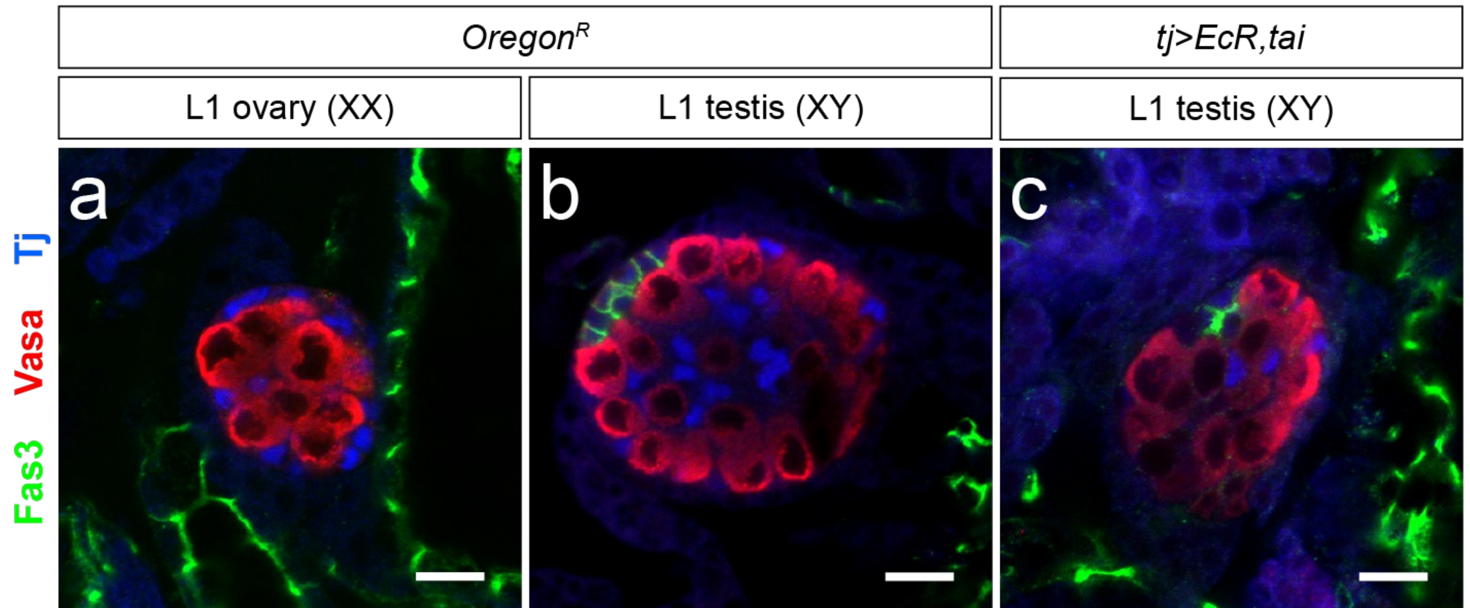
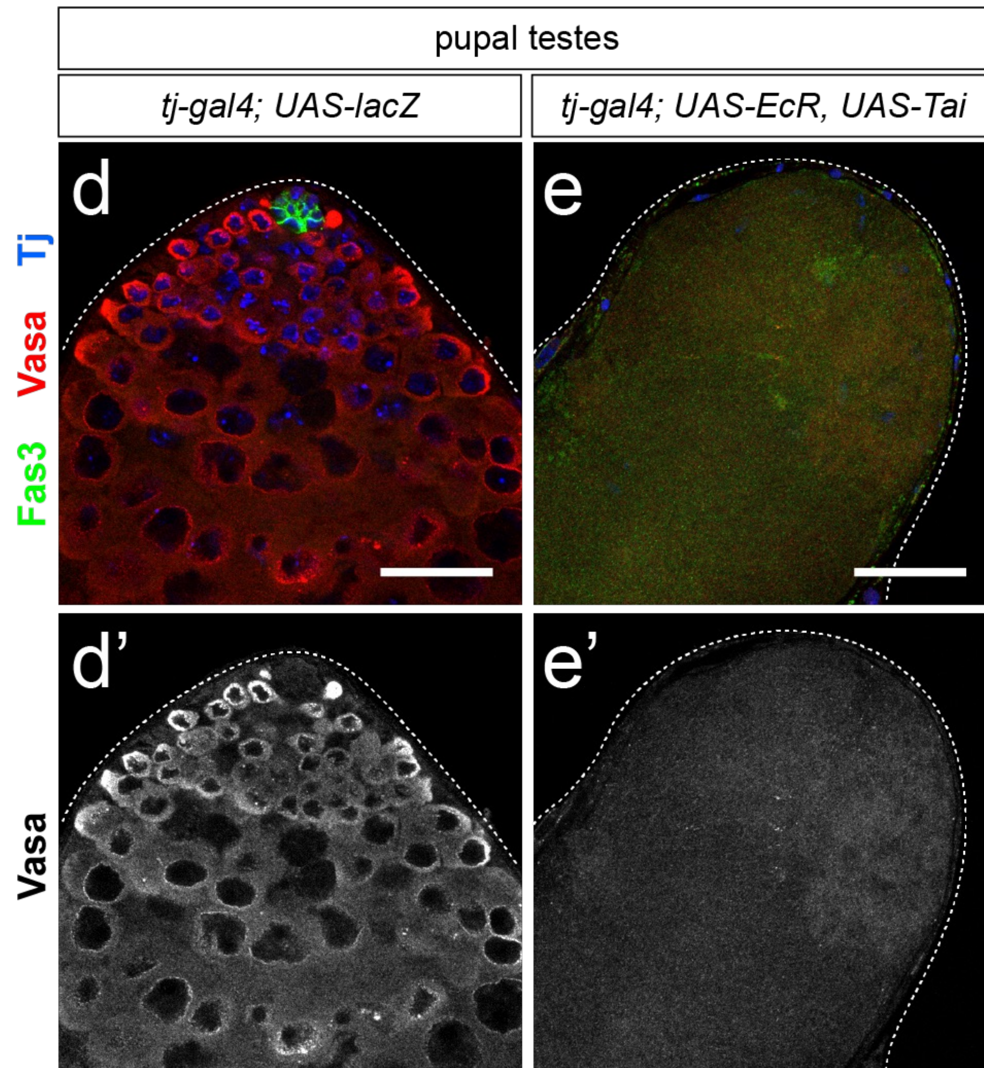
Extended Data Figure 6





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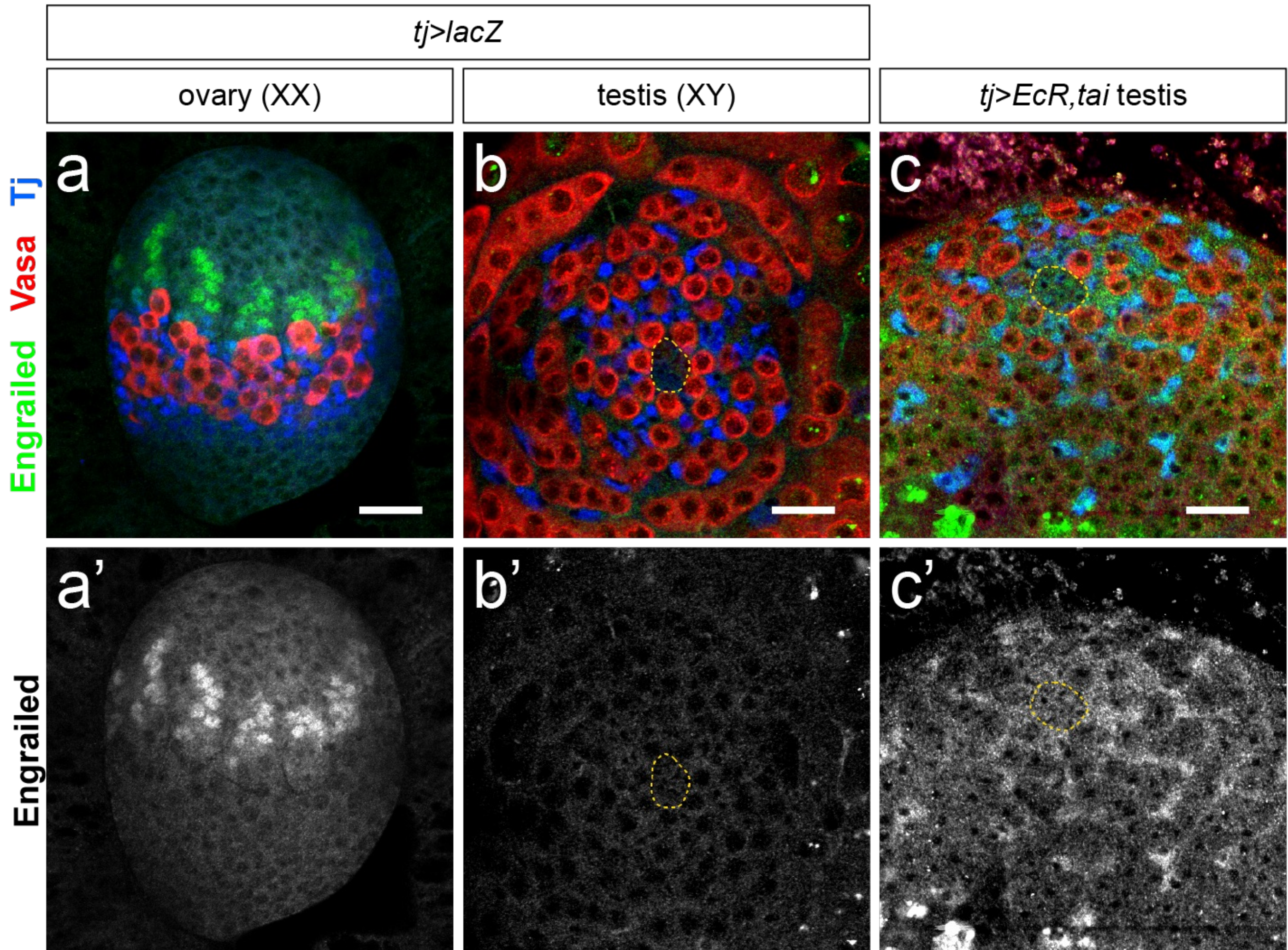
Extended Data Figure 7



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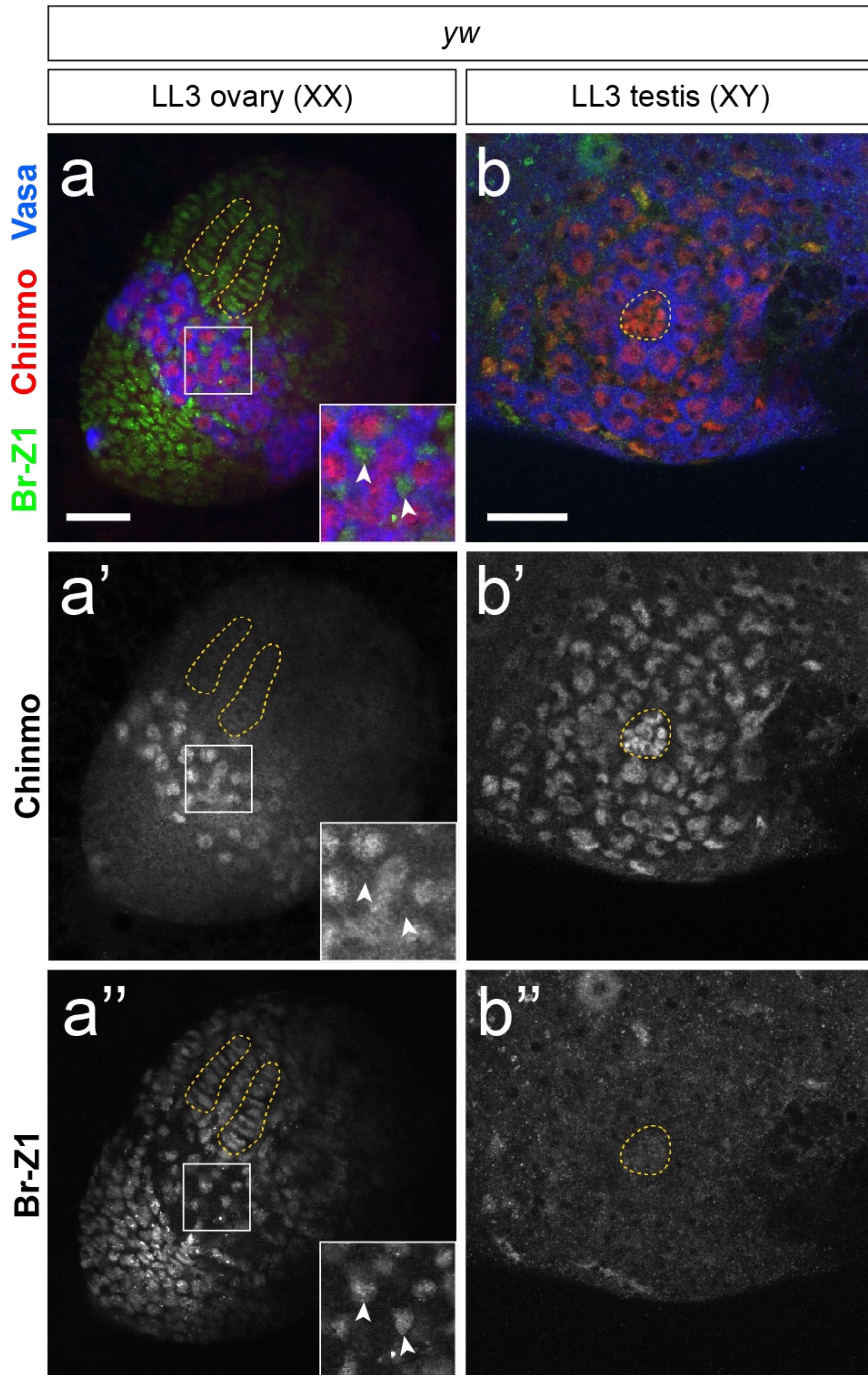
Extended Data Figure 8



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Extended Data Figure 9