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# Steroid signaling promotes stem cell maintenance in the *Drosophila* testis

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ABSTRACT

Stem cell regulation by local signals is intensely studied, but less is known about the effects of hormonal signals on stem cells. In *Drosophila*, the primary steroid twenty-hydroxyecdysone (20E) regulates ovarian germline stem cells (GSCs) but was considered dispensable for testis GSC maintenance. Male GSCs reside in a microenvironment (niche) generated by somatic hub cells and adjacent cyst stem cells (CySCs). Here, we show that depletion of 20E from adult males by overexpressing a dominant negative form of the *Ecdysone receptor* (*EcR*) or its heterodimeric partner *ultraspiracle* (*usp*) causes GSC and CySC loss that is rescued by 20E feeding, uncovering a requirement for 20E in stem cell maintenance. EcR and USP are expressed, activated and autonomously required in the CySC lineage to promote CySC maintenance, as are downstream genes *ftz-f1* and *E75*. In contrast, GSCs non-autonomously require ecdysone signaling. Global inactivation of *EcR* increases cell death in the testis that is rescued by expression of *EcR-B2* in the CySC lineage, indicating that ecdysone signaling supports stem cell viability primarily through a specific receptor isoform. Finally, *EcR* genetically interacts with the NURF chromatin-remodeling complex, which we previously showed maintains CySCs. Thus, although 20E levels are lower in males than females, ecdysone signaling acts through distinct cell types and effectors to ensure both ovarian and testis stem cell maintenance.



## Introduction

Adult stem cells, which are essential for the maintenance of many tissues, reside in niches, or local microenvironments, where distinct signals prevent their differentiation (or promote their maintenance) (de Cuevas and Matunis, 2011; Li and Xie, 2005). Stem cells can respond to both local and systemic signals including nutrition and hormones, which convey information about the organism's environment to the tissues and coordinate responses to physiological change (Drummond-Barbosa, 2008; Drummond-Barbosa and Spradling, 2001; Gancz and Gilboa, 2013; Hsu et al., 2008; Ito et al., 2004; Li and Xie, 2005; McLeod et al., 2010). Some of the best-characterized niches are found in the *Drosophila* gonads, where germline stem cells (GSCs) and supporting somatic stem cells remain active throughout adulthood, ensuring a lifetime supply of sperm or eggs (Spradling et al., 2011). However, the role of hormonal signaling in stem cell maintenance is not fully understood, especially in the *Drosophila* testis (Gancz and Gilboa, 2013).

In *Drosophila*, the steroid hormone twenty-hydroxyecdysone (20E), generated from the prohormone ecdysone, is essential for coordinating development at all stages, including embryogenesis, larval molting, puparium formation, and metamorphosis (Baehrecke, 1996; Yamanaka et al., 2013). 20E acts by binding to a heterodimeric nuclear hormone receptor complex composed of *Ecdysone receptor* (*EcR*) and *ultraspiracle* (*usp*), which are mammalian orthologues of *franesoid X receptor/liver X receptor* and *retinoid X receptor*, respectively (Hayward et al., 1999; King-Jones and Thummel, 2005). This complex binds to specific promoter sequences, called Ecdysone Response Elements (EcREs), and can activate or repress the expression of hundreds of target genes which vary in response to the presence or absence of cell-type-specific co-activators (Carbonell et al., 2013; Francis et al., 2010; Jang et al., 2009; Perera et al., 2005; Tsai et al., 1999) (Fig. 1(A)). Additional temporal and spatial control of 20E signaling is generated through alternative splicing of transcripts encoded by the *EcR* gene to yield three isoforms, *EcR-A*, *EcR-B1*, and *EcR-B2*; these receptors share common ligand binding domains (LBDs) and DNA binding domains (DBDs) but vary at their amino-termini. Each *EcR* isoform has a distinct expression pattern and response to 20E throughout development (Talbot et al., 1993).

Although ecdysone signaling has been studied primarily during metamorphosis, 20E is also present, albeit at lower levels, in adult *Drosophila* (Bownes et al., 1984; Handler, 1982; Hodgetts et al., 1977; Kozlova and Thummel, 2000). Adult 20E titers respond to changes in diet and environment (Riehle and Brown, 1999; Tu et al., 2002) and can also be modulated genetically. In this case, however, conditional manipulation of hormone levels is necessary due to

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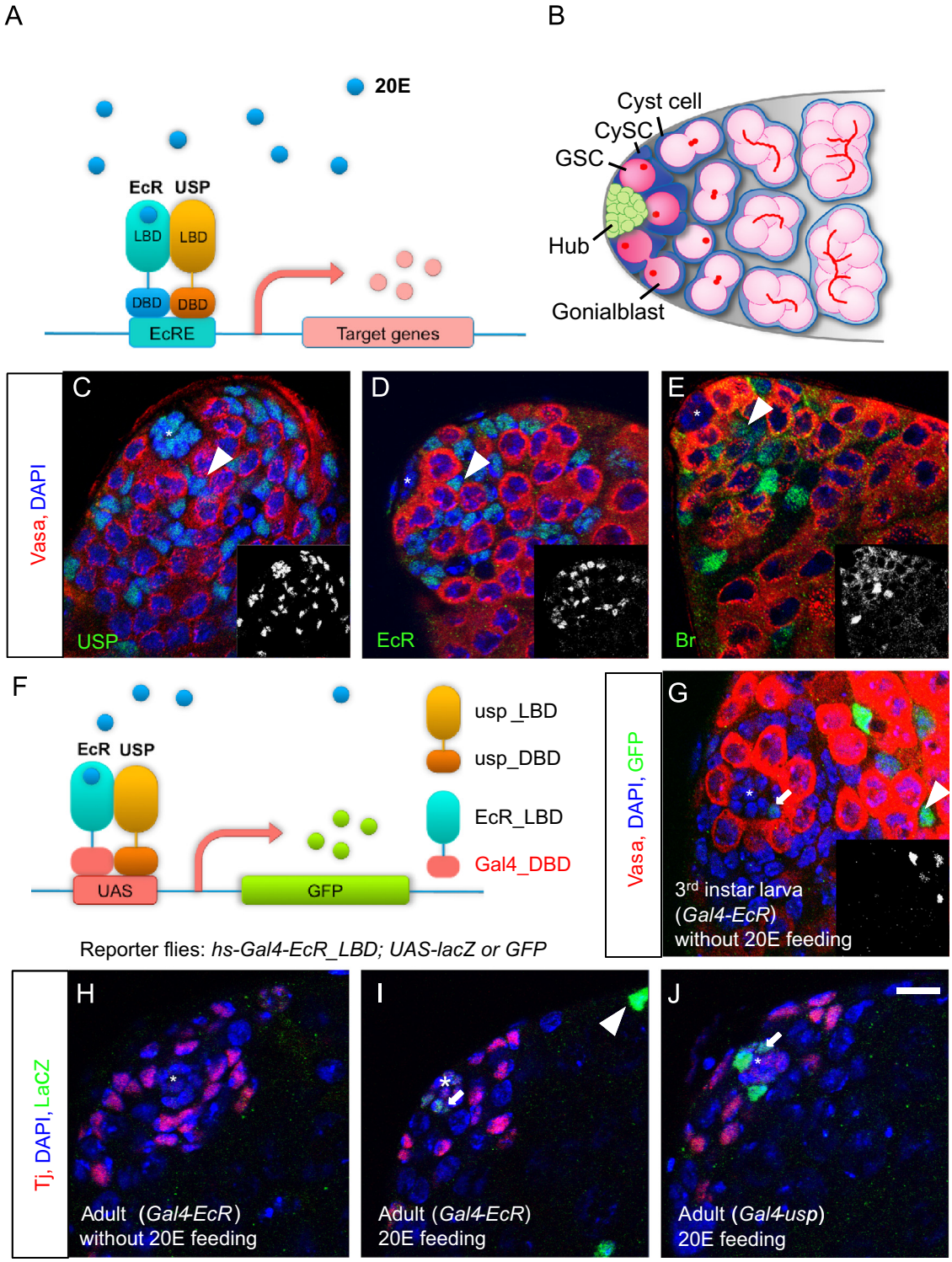


**Fig. 1.** Ecdysone signaling components are expressed and activated in the *Drosophila* testis niche. (A) Diagram of the *Drosophila* testis. Around 10 GSCs (3 shown, pink) are attached to the hub. GSCs divide asymmetrically to produce daughter gonialblasts (GB) that are displaced from the hub. GBs go on to form spermatogonial cysts. Fusomes (red) are spherical in GSCs and branched in spermatogonia. Approximately 2 CySCs (blue) flank each GSC and contact the hub with cytoplasmic extensions. CySCs divide to produce cyst cell daughters; two envelop each GB and its descendants. (B) Diagram of the *Drosophila* ecdysone pathway. 20E (blue dots) activates this pathway by binding to a heterodimer composed of EcR and USP. Both EcR and USP contain a LBD that can bind 20E and a DBD that can recognize the EcRE and regulate downstream gene expression (pink dots). (C)–(E) Testes from adult *y w* flies stained with germline marker anti-Vasa (red), DNA stain DAPI (blue), and antibodies (green) against: (C) USP (hub and CySC lineage); (D) EcR (CySC lineage); or (E) ecdysone signaling target Br (CySC lineage). Insets show green channel alone. (F) Diagram of the *GAL4-EcR* reporter construct, which is composed of the LBD from EcR fused to the DBD from Gal4 and is under control of the hsp-70 promoter. When expressed at low levels, this reporter shows where the pathway can be activated: in the presence of 20E and EcR's binding partners, Gal4 is activated and induces expression of *UAS-lacZ* or *UAS-GFP* (green dots). A similar *GAL4-usp* construct (not shown) is activated by ecdysone and USP's binding partners. (G) Late 3rd instar larval testis carrying the *GAL4-EcR* reporter and stained with DAPI (blue), anti-Vasa (red), and anti-GFP (green). Without 20E feeding, endogenous 20E drives GFP expression in the larval hub and CySC lineage. Inset shows green channel alone. (H)–(J) Adult testes stained with DAPI (blue), somatic cell marker anti-Tj (red), and anti-lacZ (green). Without 20E feeding (H), adult testes carrying the *Gal4-EcR* reporter (or *Gal4-usp* reporter, not shown) do not express lacZ. After adult flies carrying the *Gal4-EcR* reporter (I) or *Gal4-usp* reporter (J) are fed 1 mM 20E overnight, testes express lacZ in the hub and CySC lineage. Hub, asterisk or arrow; CySC lineage cells, arrowhead. Scale bar in J, for all panels, = 20 μm.

the essential roles of 20E during development. 20E feeding can also serve as a tool to increase hormone titers (Garen et al., 1977).

Although 20E has been shown to regulate a few aspects of adult *Drosophila* behavior including sleep and longevity, the effects of this hormone are best understood during female reproduction, where ecdysone signaling regulates multiple stages of oogenesis (Carney and Bender, 2000; Ishimoto and Kitamoto, 2010; Ishimoto et al., 2009; Tricoire et al., 2009). Oogenesis is initiated through asymmetric GSC divisions, and *EcR*, *usp*, and the ecdysone target gene and ETS-domain DNA-binding protein *Ecdysone-induced protein 74EF* (*E74*) are required directly in ovarian GSCs for their maintenance and proliferation. Both *EcR* and *E74* interact genetically with components of the Nucleosome remodeling factor (NURF) complex, suggesting that ecdysone signaling regulates GSCs by modulating their epigenetic state (Ables and Drummond-Barbosa, 2010). Ovarian GSCs are also regulated indirectly by ecdysone signaling: *EcR*, *usp*, and the ecdysone target and nuclear hormone receptor *Ecdysone-induced protein 75B* (*E75*) are required in the somatic escort cells of the ovary for GSC maintenance (Morris and Spradling, 2012). Ecdysone signaling is also required for many subsequent steps in oogenesis including germline differentiation, entry into meiosis, and formation and progression of egg chambers past mid-oogenesis (Buszczak et al., 1999; Konig et al., 2011; Morris and Spradling, 2012).

In contrast to the wealth of information regarding the roles of ecdysone signaling in the ovary, little is known of its requirements in male reproduction. Adult *Drosophila* males contain lower titers of 20E than females, and although the hormone has been detected in the testis (Bownes et al., 1984; Handler, 1982; Hodgetts et al., 1977; Parisi et al., 2010), ecdysone signaling was recently described as being dispensable for GSC maintenance and early germ cell development in males (Morris and Spradling, 2012). However, we previously found that the NURF complex is required for stem cell maintenance in the testis (Cherry and Matunis, 2010). In light of the physical and genetic interactions between NURF and ecdysone pathway components during development and oogenesis (Ables and Drummond-Barbosa, 2010; Badenhorst et al., 2005), we were prompted to look more closely at the role of ecdysone signaling in the testis stem cell niche.

The *Drosophila* testis stem cell niche resides in the testis apex, where a cluster of non-mitotic somatic cells called the hub produces signals that maintain surrounding GSCs and cyst stem cells (CySCs) (Fig. 1(B)). GSCs generate gonialblast daughters, which mitotically amplify and ultimately differentiate into sperm; CySCs produce non-mitotic daughters called cyst cells, two of which envelop each gonialblast and its descendants, supporting their differentiation into sperm. Here, we report that ecdysone signaling pathway components are expressed and activated in CySC lineage cells and are required directly in these cells to maintain both GSCs and CySCs, which do not survive in the absence of ecdysone signaling. Moreover, we show that *EcR* interacts genetically with *Enhancer of bithorax* (*Nurf301*), a component of the NURF complex, to maintain stem cells in the testis niche. Thus, steroid signaling is required for stem cell maintenance in both the ovary and testis of *Drosophila*, where it might act in part by regulating the epigenetic state of the stem cells.

## Results

### *Ecdysone signaling components are expressed and activated in the Drosophila testis*

To determine whether ecdysone signaling plays a role in the adult *Drosophila* testis, we began by asking whether ecdysone receptors and downstream targets of the pathway are expressed in this tissue. We used immunostaining to determine the expression patterns of *EcR*, *usp*, and the downstream targets *broad* (*br*), *E75* and *ftz transcription factor 1* (*ftz-f1*) in the testis apex. We found that USP is expressed in the hub and CySC lineage cells (Fig. 1(C)), while EcR and Br are enriched in the CySC lineage (Fig. 1(D) and (E)). Although transcripts encoding E75 and Ftz-f1 were detected in the testis by RNA-seq (Gan et al., 2010), these proteins are below the level of detection via immunostaining in adult testes (although they were detected in other tissues; data not shown). Thus, several key ecdysone pathway components are present within the testis apex, and their expression is largely confined to somatic cells.

Since ecdysone pathway members are expressed in the testis apex, we next asked which cells in this tissue actively transduce ecdysone signaling. Transgenic flies containing chimeric receptors are well-established tools for detecting ecdysone receptor complex (EcR and USP) activation within tissues. These receptors contain the ligand-binding domain from either EcR or USP fused to the yeast GAL4 DNA-binding domain (*GAL4-EcR* or *GAL4-usp*) under control of a heat-inducible promoter, which allows for precise temporal control of their expression (Kozlova and Thummel, 2002). Binding of GAL4-EcR or GAL4-USP to a second transgene encoding a reporter (lacZ or GFP) under control of an upstream activating sequence (UAS), which is recognized by the Gal4 DNA-binding domain, reveals cells with active ecdysone signaling. When flies carrying both transgenes are exposed to high temperature, chimeric receptors are expressed throughout the fly; however, UAS-reporter genes are expressed only in cells containing 20E and the cognate receptor (USP or other binding partners for *GAL4-EcR*; EcR or other binding partners for *GAL4-usp*) (Fig. 1(F)) (Kozlova and Thummel, 2002; Palanker et al., 2006). We first examined testes from late 3rd instar larvae expressing GAL4-EcR, because at this stage, the stem cell niche is fully functional but the endogenous 20E levels are higher than in adults (Hardy et al., 1979; Kozlova and Thummel, 2000). We observed weak GFP expression in a few hub cells and stronger expression in late cyst cells (Fig. 1(G)). However, when flies develop to adulthood and 20E titers have diminished (Schwedes and Carney, 2012), GFP expression is no longer detectable within the testis (Fig. 1(H)). Therefore, we hypothesized that in larval testes, endogenous 20E levels are sufficient to induce *GAL4-EcR* activation in the somatic lineage, but in adult testes, 20E availability might be a limiting factor. To test this hypothesis, we fed exogenous 20E to adult flies containing *GAL4-EcR* and *UAS-lacZ* and then examined the reporter gene expression within the testis. We found that 20E feeding caused *GAL4-EcR* activation in the hub and CySC lineage in a pattern similar to that seen in 3rd instar larval testes in response to endogenous hormone (Fig. 1(I)). We conclude that adult hub and CySC lineage cells are competent to respond to 20E via EcR, but that the levels of 20E needed to produce a detectable signal using this reporter are insufficient when flies are fed standard food. When we repeated the 20E feeding with flies expressing *GAL4-usp*, we again saw GFP expression in the hub and late cyst cells (Fig. 1(J)). We expected to see activation of these reporters in the CySC lineage, but were surprised to find GFP expression in the hub; *GAL4-usp* requires a binding partner to function, and we did not detect endogenous EcR expression in the hub (Fig. 1(D)). Perhaps low levels of EcR are present in the hub (but undetectable by immunostaining) and are sufficient to activate reporter gene expression. However, *usp*, unlike *EcR*, can signal through additional binding partners such as *Hormone receptor-like in 38* (*DHR38*) (Baker et al., 2003; Jones et al., 2001); these partners, which have not been characterized in the testis, may permit activation of *GAL4-usp*. We observed that the activation of both ecdysone activity reporters was limited to only a few cells, and we suspect that this is due to a limited supply of binding partners. In support of this idea, *GAL4-usp* activation becomes detectable in almost all hub and CySC lineage cells upon co-expression of EcR (data not shown). This finding suggests that the low levels of endogenous EcR detected by

immunostaining in the CySC lineage are insufficient to activate *GAL4-usp* in all cells. Similarly, expression of a constitutively active form of the EcR co-activator *taiman* (*tai*) yielded *GAL4-EcR* reporter activation in almost all hub and CySC lineage cells in the testis apex (data not shown). Taken together, our results indicate that EcR and USP can be activated specifically within hub cells and CySC lineage cells in the presence of their binding partners in both larval and adult testes, and that receptor complex activation in the adult testis is ligand-dependent.

### *20E is required for male germline and somatic stem cell maintenance*

Since ecdysone signaling components are expressed and can be activated in the testis, we hypothesized that 20E plays a role in this tissue even though its endogenous titer is very low. To test this hypothesis, we asked whether 20E is required to maintain adult male GSCs or CySCs. To reduce the effective concentration of 20E, we used the *GAL4-EcR* and *GAL4-usp* constructs described above, which have been widely used as dominant negative (DN) receptors when overexpressed for an extended period of time (Hackney et al., 2007; Konig et al., 2011; Kozlova and Thummel, 2002, 2003). For example, both heat-shocked *Gal4-EcR* flies and flies expressing *UAS-EcR.B1-ΔC655.F645A*, a DN form of *EcR*, in border cells develop a similar thin eggshell phenotype (Hackney et al., 2007). Testes from control flies, which carry the *GAL4-EcR* or *GAL4-usp* construct but are un-induced, appear normal (Fig. 2(B) and (S1A)). After extended overexpression of either construct, however, testes lose most of their GSCs, early germline cells, and CySCs (Fig. 2(C), (E) and (S1B)), suggesting that signaling via 20E contributes to the

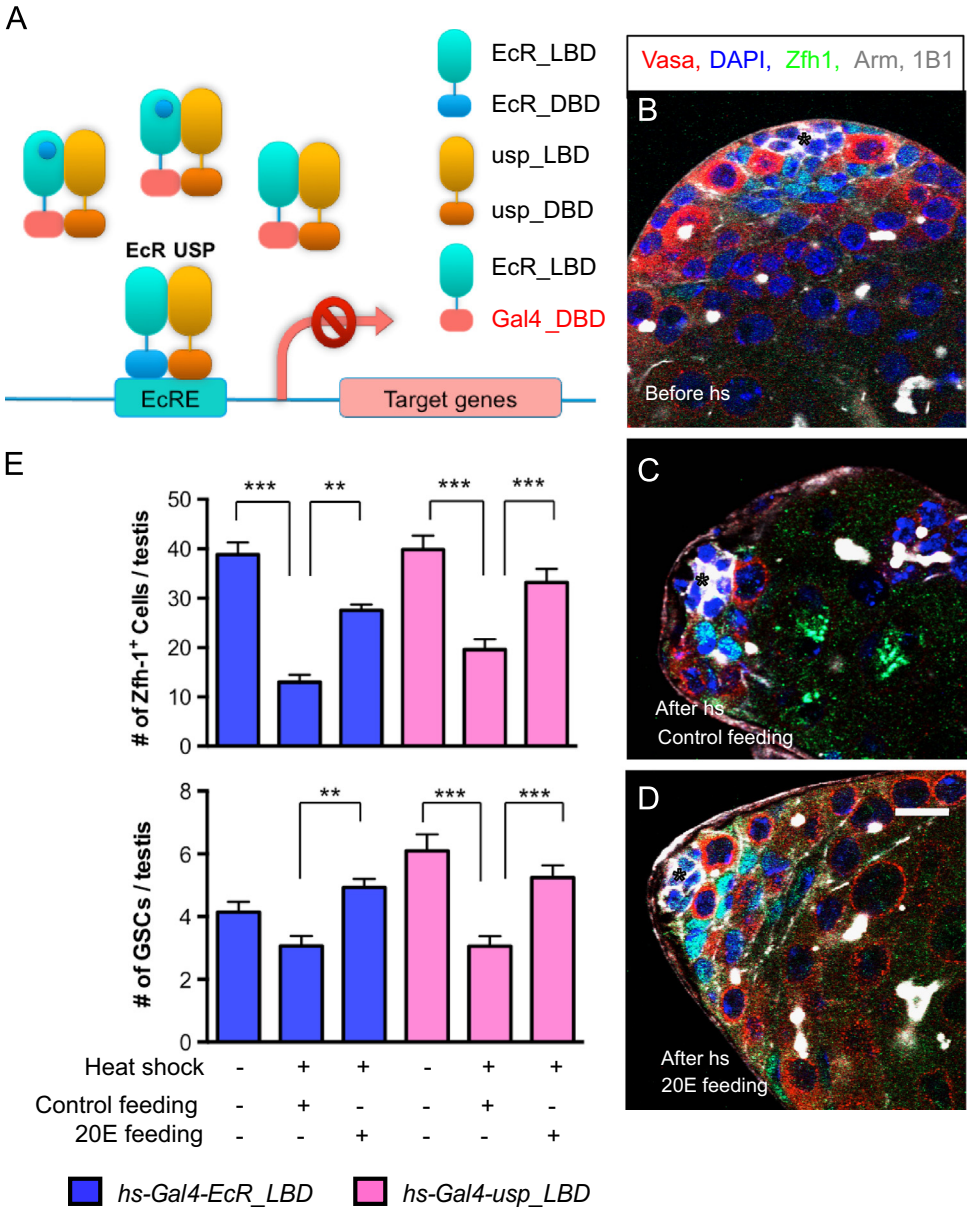


**Fig. 2.** 20E hormone is required for stem cell maintenance. (A) Diagram showing how *Gal4-EcR* or *Gal4-usp* can act as dominant negative constructs (20E "sponges"): when expressed at high levels, they bind with endogenous receptors, compete for endogenous 20E and reduce its effective concentration, thus preventing endogenous EcR or USP from functioning normally (Hackney et al. 2007). (B)–(D) Testes from adult flies carrying *Gal4-EcR* stained with anti-Vasa (red), DAPI (blue), anti-Zfh1 (green; CySCs and their immediate daughters), anti-Hts/1B1 (white; fusomes), and anti-Arm (white; hub cells). Before overexpression (B), testes look normal; after heat-shock induced overexpression of *Gal4-EcR* (C), GSCs and CySCs are lost; feeding 20E to adult flies rescues the loss (D). Scale bar in D, for B–D, = 20 μm. (E) Bar graphs showing number of GSCs or Zfh1-positive cells per testis for this experiment. Data are represented as mean ± standard error of the mean (SEM). ** *P*-value < 0.005; *** *P*-value < 0.0005.

maintenance of both stem cell populations in the testis. Because the endogenous titer of 20E in the adult testis is very low, we speculated that these constructs could act as DN receptors by binding with endogenous receptors and then competing with endogenous heterodimers for the limited amount of 20E, similar to a 20E "sponge" (Fig. 2(A)). To ask whether the loss of stem cells is due to reduced titers of 20E by *GAL4-EcR* or *GAL4-usp*, we repeated the above experiment but added 20E to the fly food to increase hormone levels. We expected that if 20E is no longer the limiting factor, endogenous *EcR* and *usp* should function normally; therefore, feeding 20E should rescue the phenotype caused by overexpression of *GAL4-EcR* or *GAL4-usp*. Consistent with our hypothesis, 20E feeding significantly rescued the GSC and CySC loss caused by extended overexpression of *GAL4-EcR* or *GAL4-usp* (Fig. 2(D), (E) and (S1C)). We conclude that although 20E is present only at very low levels in the testis, it is required to maintain GSCs and CySCs.

### *ecd plays an ecdysone-independent role in GSC and CySC maintenance*

As an alternate approach to reducing ecdysteroid levels in the testis, we used a temperature-sensitive allele of *ecdysoneless* (*ecd*$^{1}$). This steroid-deficient fly strain has long been used to study the effects of ecdysone signaling in *Drosophila*, but it has both ecdysone-dependent and independent functions (Ables and Drummond-Barbosa, 2010; Claudius et al., 2014; Garen et al., 1977; Gaziova et al., 2004). Therefore, rescue of *ecd* phenotypes by 20E feeding is important to distinguish between these possibilities. After shifting adult *ecd*$^{1}$ flies to the non-permissive temperature for 7 days, we found that their testes contained significantly fewer GSCs than unshifted control testes. We expected that we could rescue this GSC loss phenotype by feeding 20E to the flies. However, we found that the phenotype was not rescued by 20E feeding (Fig. S2(A)–(D)), although the same feeding paradigm was sufficient to activate *GAL4-EcR* (Fig. 1I and J). We conclude that *ecd*-dependent GSC loss is caused by an ecdysone-independent role of *ecd*. Moreover, mosaic analysis revealed that *ecd* is required cell-autonomously in the GSCs and CySCs for their maintenance (Fig. S2 (E), Table S1). The inability of adjacent wild-type cells to compensate for loss of *ecd* function further indicates that ecdysteroid production is not the main role for *ecd* in the testis niche. We conclude that *ecd* is required to maintain GSCs and CySCs in the testis niche; however, since its requirement is independent of 20E, *ecd* is not a useful tool for studying the role of ecdysone signaling in this tissue.

### *EcR and usp are required in the CySC lineage to maintain GSCs and CySCs*

Knowing that 20E is required to maintain stem cells in the testis, we next asked whether the ecdysone receptors *EcR* and *usp* are also required. Flies carrying a temperature sensitive allele of *EcR*, *EcR*$^{A483T}$, in trans with a null allele, *EcR*$^{M554fs}$, have normal numbers of GSCs and CySCs when raised at permissive temperature (Fig. 3B). However, after 7 days at restrictive temperature, *EcR*$^{A483T/M554fs}$ (*EcR*$^{ts}$) flies have significantly fewer GSCs and CySCs than heterozygous control flies under the same conditions (Fig. 3(A)–(D)). In addition, we found differentiating spermatogonial cells next to the hub in 23% of mutant testes at restrictive temperature ($n=31$); this phenotype, which does not occur in wild-type testes (Fig. 3(C)), is indicative of GSC depletion.

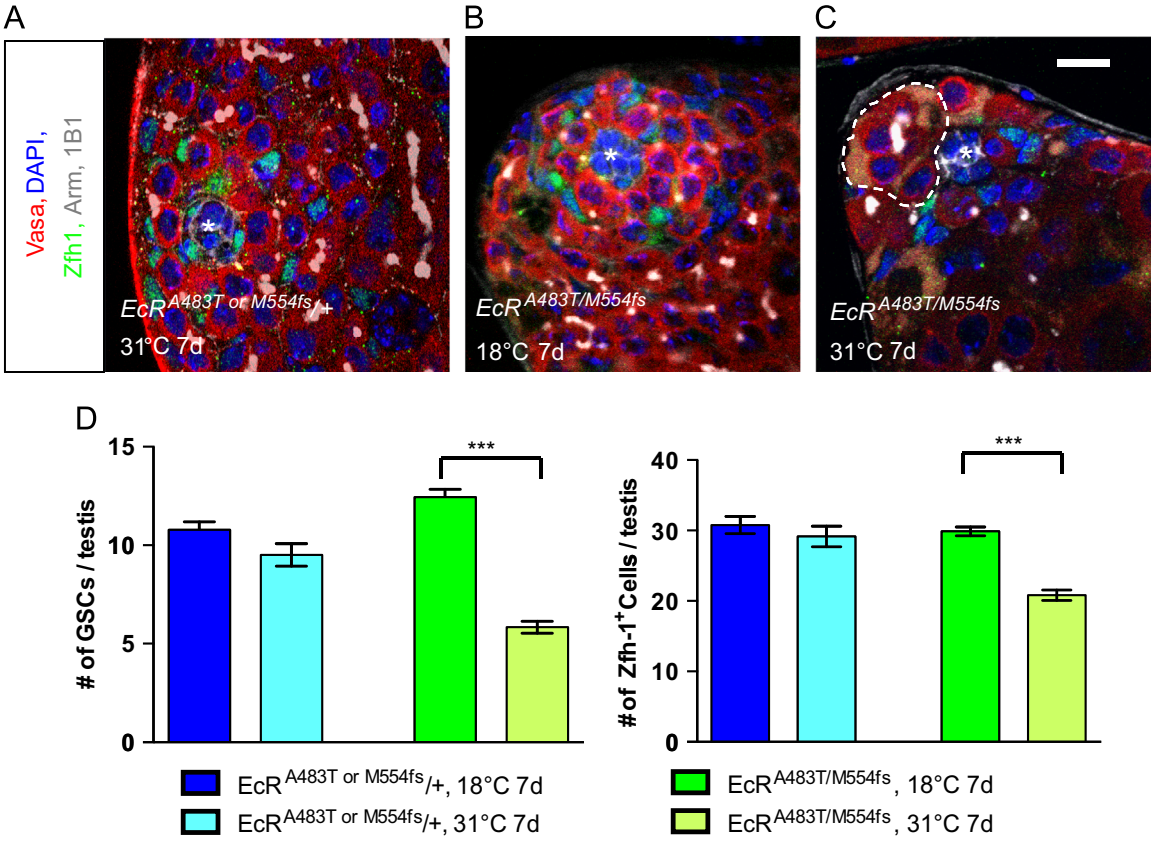


**Fig. 3.** *EcR* is required in the testis to maintain GSCs and CySCs. Testes from adult flies stained with anti-Vasa (red), DAPI (blue), anti-Zfh1 (green), anti-Hts/1B1, and anti-Arm. (A) *EcR* heterozygous mutant testes look wild-type after 7 days at 31 °C. *EcR*$^{A483T/M554fs}$ mutant testes look normal at permissive temperature (B) but lose GSCs and CySCs after 7 days at restrictive temperature (C). At restrictive temperature, spermatogonial cysts are sometimes found touching the hub; a 8-cell cyst (identified by elongated fusome) is outlined. Hub, asterisk. Scale bar in C, for A–C, = 20 μm. (D) Bar graphs showing number of GSCs or Zfh1-positive cells per testis for this experiment. Data are represented as mean ± SEM. *** *P*-value < 0.0005.

The stem cell loss phenotype of $EcR^{ts}$ testes shows that *EcR* promotes stem cell maintenance in the testis, but does not reveal which cells autonomously require *EcR*, since this mutant combination yields a global reduction in receptor activity. Since EcR and USP are undetectable in germ cells but are present in the CySC lineage, we hypothesized that these receptors are required autonomously within somatic stem

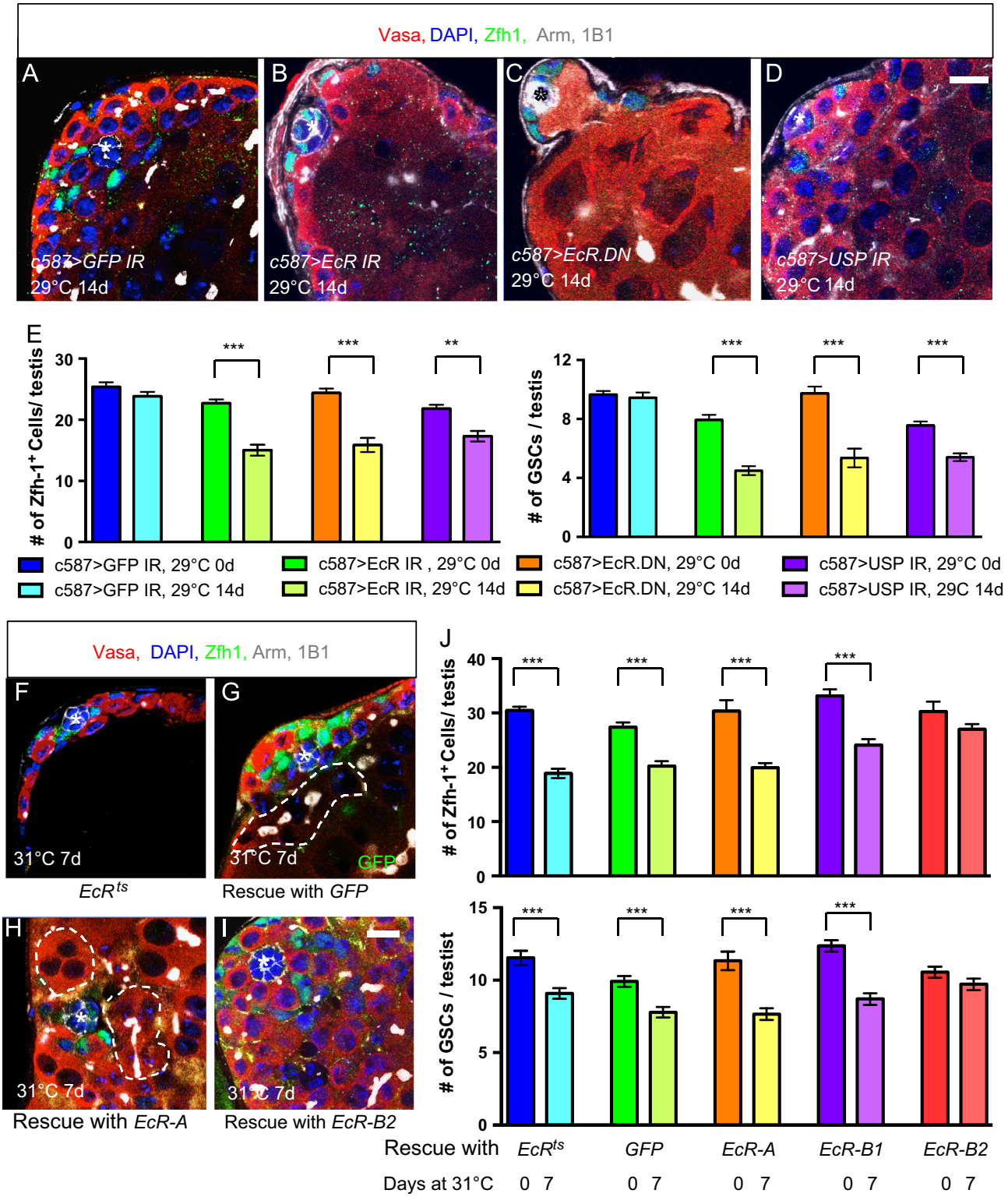


**Fig. 4.** *EcR* and *usp* are required in the CySC lineage to maintain CySCs and GSCs. (A)–(D), (F)–(I) Testes from adult flies stained with anti-Vasa (red), DAPI (blue), anti-Zfh1 (green), anti-Hts/1B1, and anti-Arm. (A) Mock disruption of GFP by RNAi in the CySC lineage does not affect GSC or CySC maintenance. (B)–(D) Disruption of EcR by RNAi (B) or DN (C) or disruption of USP by RNAi (D) in the CySC lineage causes loss of GSCs, early germline cells, and CySCs. (E) Bar graphs showing the number of Zfh1-positive cells or GSCs per testis for the experiments depicted in panels A–D. (F) Testes from *c587-Gal4*; $EcR^{A483T/M554fs}$; *TM6B/+* flies lose GSCs and CySCs at restrictive temperature (similar to Fig. 3(C)). Expression of *UAS-EcR-B2* (I) but not *UAS-GFP* (G) or *UAS-EcR-A* (H) in the CySC lineage is able to rescue the stem cell loss phenotype in $EcR^{ts}$ testes. Outlined cells are differentiated spermatogonia near the hub. Hub, asterisk. Scale bars in D, for A–D; in I, for *F–I* = 20 μm. (J) Bar graphs showing the number of Zfh1-positive cells or GSCs per testis for the experiments depicted in panels F-I. In (E) and (J), data are represented as mean ± SEM. ** *P*-value < 0.005; *** *P*-value < 0.0005.

cells for their maintenance. The genomic location of *usp* (on the X chromosome) and *EcR* (very close to the centromere) precludes mosaic analysis of these genes in the testis. However, RNAi-mediated knockdown is a feasible alternative. We used the CySC and early cyst-cell driver *c587-Gal4* in combination with a temperature-sensitive allele of the *Gal4* repressor *Gal80* to conditionally express transgenic RNAi or DN constructs of *EcR* or *usp* specifically in the adult testis. After 14 days of transgene induction at 29 °C, we observed a significant decrease in the number of CySCs in all four experimental genotypes (Fig. 4A E, S3 and S4). Although we could not detect EcR in the hub, we did detect USP there, so we also asked whether there is a requirement for each receptor in hub cells. However, hub cells in testes containing RNAi-mediated knock down of *EcR* or *usp* in the hub were indistinguishable from those in control testes; in addition there was no significant effect on CySC numbers (Fig. S4). These results indicate that *EcR* and *usp* are cell-autonomously required in the CySC lineage, but not in hub cells, for CySC maintenance. After *EcR* or *usp* knockdown in the CySC lineage, we also found that the number of GSCs decreased significantly (Fig. 4E), which suggests that *EcR* and *usp* are required indirectly in the CySC lineage for GSC maintenance. GSCs could be lost simply as a consequence of CySC loss, but it is also possible that they rely on ecdysone-dependent maintenance signals from CySCs. We have never observed expression or activation of ecdysone signaling pathway components in GSCs, or significant GSC loss, when *EcR* or *usp* are knocked down by RNAi in the germline (data not shown). We conclude that *EcR* and *usp* are required autonomously in the CySC lineage, and non-autonomously for GSC maintenance.

We next asked whether expression of EcR only in the CySC lineage is sufficient to rescue the stem cell loss phenotype of $EcR^{ts}$ testes and whether the requirement of *EcR* is isoform-specific. To answer this question, we expressed each isoform (*EcR-A*, *EcR-B1*, or *EcR-B2*) independently in the CySC lineage in the $EcR^{ts}$ mutant background. Interestingly, we found that expression of *EcR-B2*, but not *EcR-A* or *EcR-B1*, in the CySC lineage is able to fully rescue the $EcR^{ts}$ stem cell loss phenotype (Fig. 4F J). In contrast, expression of *EcR-A*, *EcR-B1*, or *EcR-B2* in hub cells did not rescue the $EcR^{ts}$ phenotype (Fig. S5). These results indicate that within the CySC lineage, *EcR* is necessary for stem cell maintenance in the testis, and its requirement is specific to the *EcR-B2* isoform, which can act as a strong ligand-dependent transcriptional activator (King-Jones and Thummel, 2005).

### *EcR is required for cell survival in the testis*

Ecdysone signaling is known to regulate apoptosis during development, and in the ovary, developing germline cysts lacking ecdysone signaling die more often than control cysts (Ables and Drummond-Barbosa, 2010; Zirin et al., 2013). Therefore, we asked whether stem cell loss in $EcR^{ts}$ testes at restrictive temperature could be caused by increased cell death. We used terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) to detect fragmented DNA in dying

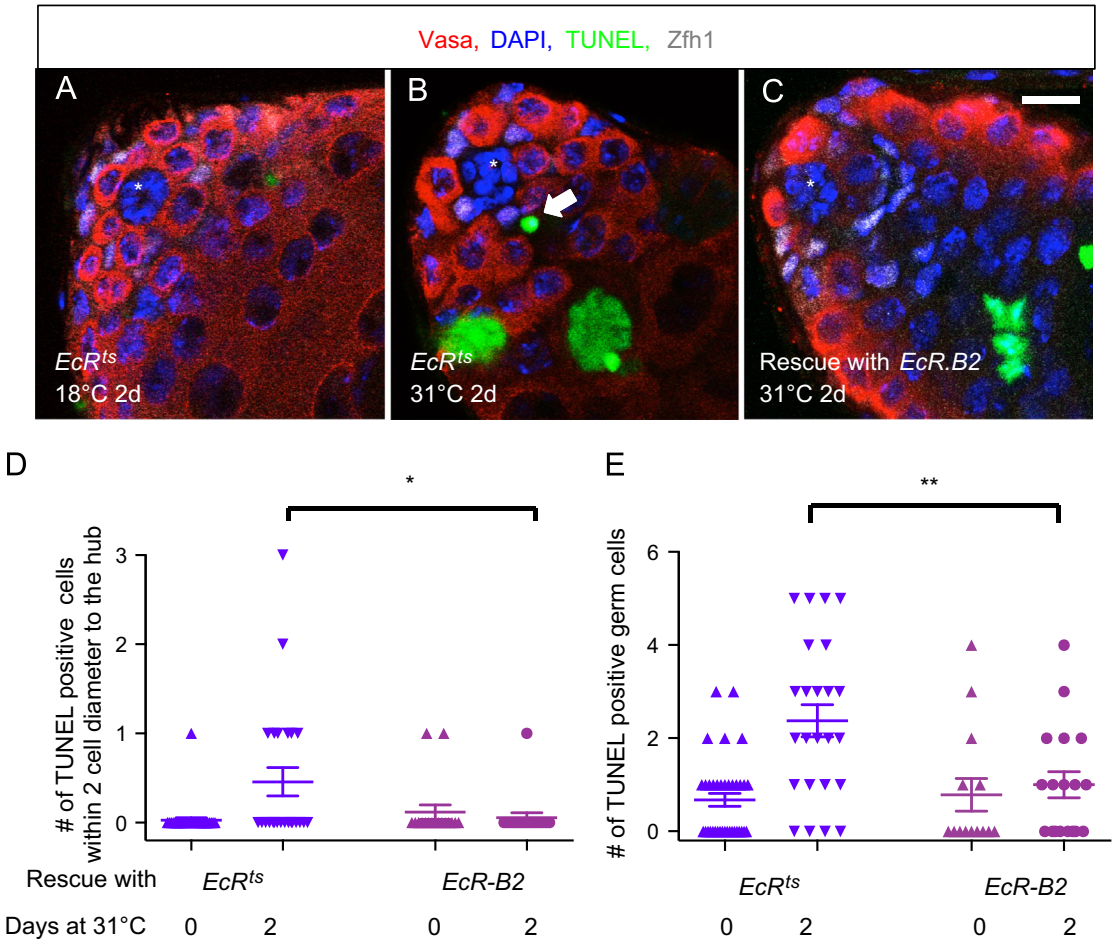


**Fig. 5.** *EcR* is required for cell survival in the testis. Testes from adult flies stained with anti-Vasa (red), DAPI (blue), anti-Zfh1, and TUNEL (green) to visualize apoptotic cells. *c587-Gal4*; $EcR^{A483T/M554fs}$; *TM6B/+* testes contain more TUNEL-positive cells at restrictive temperature (B) than at permissive temperature (A). TUNEL-positive cells are rarely found within 2 cell diameters of the hub under normal conditions, but their number increases at restrictive temperature (arrowhead) suggesting that *EcR* is required for early cell survival in the testis. (C) Expression of *UAS-EcR.B2* in the CySC lineage is able to rescue the increased cell death phenotype in the $EcR^{ts}$ testes. Hub, asterisk. Scale bar in C, for A–C, = 20 μm. (D, E) Column scatter graphs showing the number of TUNEL-positive cells within 2 cell diameters of the hub (D) and the number of TUNEL-positive germ cells (diameter > 5 μm) (E) per testis for these experiments. Bars indicate mean ± SEM. * *P*-value < 0.05; ** *P*-value < 0.005.

cells, and we counted the number of dying cells in testes from $EcR^{ts}$ flies that remained at permissive temperature (control testes) or were shifted to restrictive temperature for 2 days. As expected, dying cells were rarely found within the stem cell zone (within 2 cell diameters of the hub) in control testes, but in testes at restrictive temperature we observed significantly more of them (Fig. 5 A, B and D). These testes also had significantly more germ cell death in the differentiating cell zone than did control testes (Fig. 5E). To confirm that the increase in cell death is due to dysfunction of *EcR*, we expressed the *EcR-B2* isoform in the CySC lineage in $EcR^{ts}$ testes at restrictive temperature and found that it rescues the increased cell death phenotype (Fig. 5C E). Taken together, these results suggest that *EcR-B2* in the CySC lineage is necessary for promoting cell survival in the testis stem cell niche. However, it is possible that GSCs and CySCs are also lost due to early differentiation of stem cells.

### *The 20E targets E75 and ftz-f1, but not br, promote stem cell maintenance in the testis*

Ecdysone signaling is mediated by multiple target genes which vary by tissue type and developmental stage (Andres and Thummel, 1992). To identify potential 20E target genes in the adult testis niche, we surveyed testis RNA-seq data for the expression of known ecdysone-responsive genes (RPKM > 1 in wild type testes) (Gan

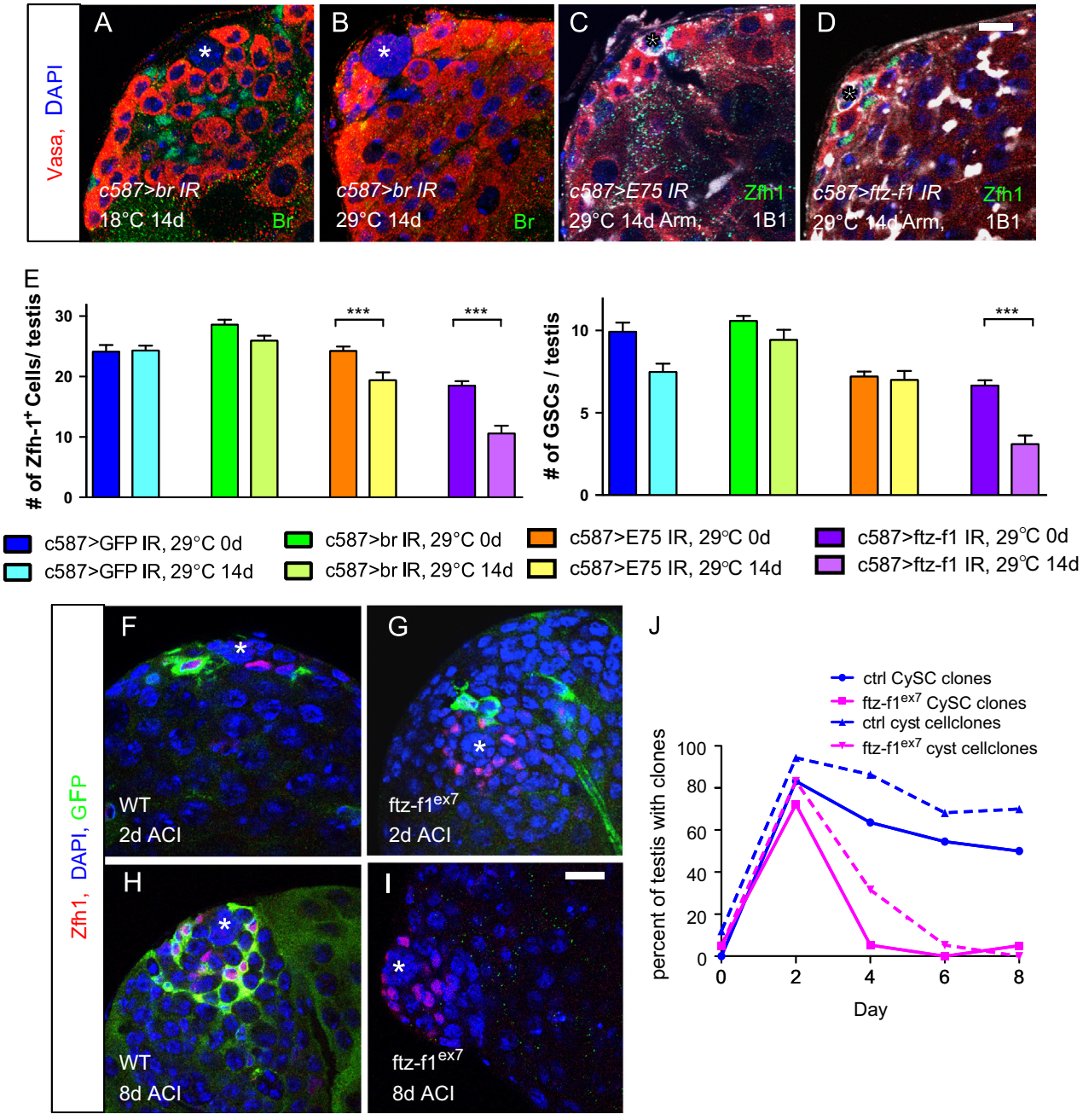


**Fig. 6.** *E75* and *ftz-f1*, but not *br*, are potential ecdysone targets that regulate stem cell maintenance in the testis. (A) and (B) Testes from adult flies stained anti-Vasa (red), DAPI (blue), anti-Br (green). Disruption of Br by RNAi in the CySC lineage can effectively reduce Br level by immunostaining, but it does not cause GSC or CySC loss. (C) and (D) Testes from adult flies stained with anti-Vasa (red), DAPI (blue), anti-Zfh1 (green), anti-Hts/1B1, and anti-Arm. Disruption of *E75* (C) or *ftz-f1* (D) by RNAi in the CySC lineage causes CySC and GSC loss. (E) Bar graphs showing the number of Zfh1-positive cells or GSCs per testis for these experiments. Data are represented as mean ± SEM. *** *P*-value < 0.0005. (F)–(I) Testes from adult flies stained with anti-GFP (green), DAPI (blue), anti-Zfh1 (red). *ftz-f1*$^{ex7}$ CySC (GFP positive, Zfh1 positive) and cyst clones (GFP positive, Zfh1 negative) are induced at a similar rate but lost faster than wild type clones. ACI, after clone induction.

**Table 1**
*ftz-f1* is required cell autonomously for GSC and CySC maintenance.

| *Part A ftz-f1* negative clonal analysis | | | | |
|---|---|---|---|---|
| Genotype | 0d ACI | 2dACI | 6dACI | 8dACI |
| Percentage of testis with CySC clones | | | | |
| *ftz-f1$^{ex7}$ FRT2A* | 17% (3/18) | 17% (7/30) | 7% (2/30) | 5% (1/20) |
| *Ctrl FRT2A* | 0% (0/17) | 77% (26/34) | 40%(6/15) | 35% (10/29) |

| Part B *ftz-f1* MARCM Genotype | 0d ACI | 2d ACI | 4d ACI | 6d ACI | 8d ACI |
|---|---|---|---|---|---|
| Percentage of testes with CySC clones | | | | | |
| *ftz-f1$^{ex7}$ FRT2A* | 5% (1/21) | 72% (13/18) | 5% (1/22) | 0% (0/24) | 5% (1/23) |
| *Ctrl FRT2A* | 0% (0/25) | 83% (15/18) | 63% (14/22) | 55% (12/22) | 50% (10/22) |
| Percentage of testis with cyst cell clones | | | | | |
| *ftz-f1$^{ex7}$ FRT2A* | 5% (1/21) | 83% (15/18) | 32% (7/22) | 5% (1/24) | 0% (0/23) |
| *Ctrl FRT2A* | 12% (3/25) | 94% (17/18) | 86% (19/22) | 68% (15/22) | 70% (14/20) |

et al., 2010), espeically those with known requirements in other adult stem cell-based tissues, including the ovary and intestine (Ables and Drummond-Barbosa, 2010; Gan et al., 2010; Morris and Spradling, 2012; Zeng and Hou, 2012). Using these criteria, we found three candidate ecdysone targets, *E75*, *ftz-f1* and *br*, and tested the requirement for each gene in CySC maintenance using RNAi-mediated knockdown in the CySC lineage. We found that *E75* or *ftz-f1* knockdown causes a loss of GSCs and CySCs that is similar to the phenotype resulting from knockdown of *EcR* or *usp* (Fig. 6C–E). In contrast, knockdown of *br* in the CySC lineage shows no effect on stem cell maintenance even though we observed significant reduction of Br protein level in the cyst stem cell lineage, confirming the efficacy of *br* knockdown (Fig. 6A, B and E). Mosaic analysis of *E75* and *ftz-f1* confirmed that these two factors are cell autonomously required for CySC maintenance (Fig. 6F J, Tables 1 and 2). We conclude that the 20E target genes *E75* and *ftz-f1,* but not *br,* are required for CySC maintenance.

### *EcR genetically interacts with Nurf301 to maintain stem cells in the testis*

In the *Drosophila* ovary, *EcR* interacts genetically with *Nurf301*, which encodes a component of the NURF chromatin remodeling complex, to promote GSC maintenance (Ables and Drummond-Barbosa, 2010). Since we had previously found that NURF is also autonomously required to promote the maintenance of male GSCs and CySCs (Cherry and Matunis, 2010), we wondered if *EcR* and *Nurf301* function together in the testis. To test this hypothesis, we asked whether reduced *Nurf301* expression levels could enhance the stem cell loss phenotype of EcR knockdown. We accomplished this by knocking down *EcR* expression specifically in the CySC lineage in a *Nurf301* heterozygous background. *Nurf301* heterozygous mutant testes are indistinguishable from wild-type testes and have normal numbers of GSCs and CySCs (Cherry and Matunis, 2010). In contrast, reducing *EcR* expression in *Nurf301* heterozygous CySCs causes a significant reduction in the number of GSCs and CySCs (Fig. 7). This result suggests that the ecdysone signaling pathway functions together with the NURF chromatin-remodeling complex to promote stem cell maintenance in both the ovary and the testis.

## Discussion

Our work shows that the steroid hormone 20E plays an important role in maintaining stem cells in the *Drosophila* testis: 20E, receptors of ecdysone signaling, and downstream targets are required directly in CySCs for their maintenance. When ecdysone signaling is lost in CySCs, GSCs are also lost, but it is unclear if their maintenance requires an ecdysone-dependent or independent signal from the CySCs. We also show that the requirement for EcR in the testis is isoform-specific: expression of *EcR-B2* in the CySC lineage is sufficient to rescue loss of GSCs and CySCs and increased cell death in *EcR* mutant testes, suggesting that there might be a temporal and spatial control of ecdysone signaling in the adult testis. In addition, we provide evidence that ecdysone signaling, as in the ovary, is able to interact with an intrinsic chromatin-remodeling factor, *Nurf301*, to promote stem cell maintenance. Therefore, our studies have revealed a novel role for ecdysone signaling in *Drosophila* male reproduction.

**Table 2**
*E75* clonal analysis indicates that *E75* is cell autonomously required for GSC and CySC maintenance.

| Genotype | 0d ACI | 2d ACI | 8d ACI |
|---|---|---|---|
| Percentage of testis with CySC clones | | | |
| *E75$^{\Delta 51}$ FRT80B* | 10% (2/20) | 30% (7/23) | 11% (4/35) |
| *Ctrl FRT80B* | 9% (2/22) | 36% (9/25) | 25% (7/29) |

### *Hormone signaling in the ovary and testis*

Although ecdysone signaling is required in both ovaries and testes for stem cell maintenance, the responses in each tissue are likely to be sex-specific. In the ovary, 20E controls GSCs directly, by modulating their proliferation and self-renewal, and it acts predominantly through the downstream target gene *E74* (Ables and Drummond-Barbosa, 2010). In contrast, male GSCs require ecdysone signaling only indirectly: we found that ecdysone signaling is required in the CySC lineage to maintain both CySCs and GSCs. In a previous study, RNAi-mediated knockdown of *EcR*, *usp* or *E75* in the CySC lineage did not result in a significant loss of GSCs (Morris and Spradling, 2012); however, the number of CySCs was not determined, and the phenotype was examined after 4 or 8 days, not 14 days as in our study. We suspect that the earlier time points used in that study may not have allowed enough time for a significant number of GSCs to be lost.

### *Spatial and temporal regulation of ecdysone signaling*

During development, 20E is produced in the prothoracic gland (PG) and further metabolized to 20E in target tissues, but the PG does not persist into adulthood (Gilbert et al., 2002; Huang et al., 2008).

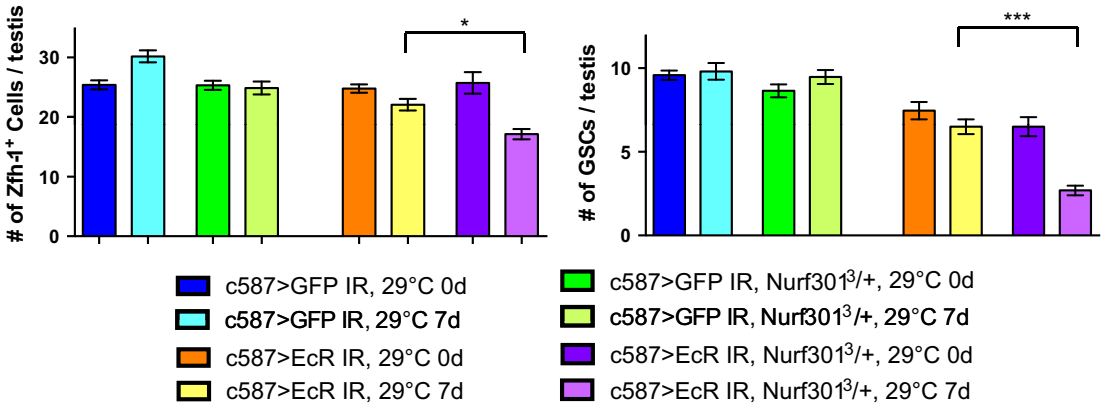


**Fig. 7.** *EcR* genetically interacts with *Nurf301* to maintain stem cells in the testis. Induction of *EcR RNAi* (7 days at 29 °C) in the CySC lineage using *c587-Gal4* driver causes GSC and CySC loss; in a *Nurf301*$^3$/+ heterozygous background, the loss is enhanced. Data are represented as mean ± SEM. * *P*-value < 0.05; *** *P*-value < 0.0005.

In adult female *Drosophila*, the ovary is a source of 20E (Schwartz et al., 1985). In contrast, the identification of steroidogenic tissues in adult male *Drosophila* remains the subject of active investigation. The level of 20E in adult males is significantly lower than in adult females, but it can be detected in the testis (Bownes et al., 1984; Handler, 1982; Hodgetts et al., 1977; Schwedes and Carney, 2012). Furthermore, RNA-seq data show that *shade*, which encodes the enzyme that metabolizes the prohomone ecdysone to 20E, is expressed in the adult testis, suggesting that the adult testis may produce 20E (Gan et al., 2010; Petryk et al., 2003). However, the sources of 20E production in adult *Drosophila* males remain to be determined experimentally.

20E, like other systemic hormones, can have tissue-specific effects or differential effects on the same cell type as development proceeds. These differences are mediated at least in part by the particular downstream target genes that are activated in each case. For example, in female 3$^{rd}$ instar larval ovaries, ecdysone signaling upregulates *br* expression to induce niche formation and PGC differentiation, but *br* is not required for GSC maintenance in the adult ovary (Gancz et al., 2011); instead, *E74* plays this role (Ables and Drummond-Barbosa, 2010). Similarly, *br* is required for the establishment of intestinal stem cells (ISCs) in the larval and pupal stages but not for ISC function in adults (Zeng and Hou, 2012). Here, we show that ecdysone signaling in the adult testis is mediated by different target genes than in the ovary: *E74*, but not *E75* or *br*, regulate stem cell function in the ovary, whereas *E75* and *ftz-f1* are important for stem cell maintenance in the testis. Since E75 is itself a nuclear hormone receptor that responds to the second messenger nitric oxide (Caceres et al., 2011; Reinking et al., 2005), it will be interesting to know whether E75's partner DHR3 also plays a role in CySCs. An intriguing question for future studies will be how different ecdysone target genes interact with the various signaling pathways that maintain stem cells in the ovary or testis.

### *Environmental changes, stem cells and hormonal signals*

Since 20E levels can actively respond to physiological changes induced by environmental cues, it is possible that the effect of 20E on testis stem cell maintenance might reflect changes in diet, stress, or other environmental cues. For example, in *Aedes aegypti*, ecdysteroid production in the ovary is stimulated by blood feeding and this is an insulin-dependent process (Riehle and Brown, 1999). In *Drosophila*, ecdysone signaling is known to interact with the insulin pathway in a complex way. Ovaries from females with hypomorphic mutations in the insulin-like receptor have reduced levels of 20E (Tu et al., 2002). Furthermore, ecdysone signaling can directly inhibit insulin signaling and control larval growth in the fat body (Colombani et al., 2005). Thus, ecdysone signaling may interact with insulin signaling during testis stem cell maintenance. Previously, it was shown that GSCs in the ovary and testis can respond to diet through insulin signaling, which is required to promote stem cell maintenance in both sexes (Drummond-Barbosa and Spradling, 2001; Flatt et al., 2008; McLeod et al., 2010; Roth et al., 2012; Ueishi et al. 2009; Wang et al., 2011). It is possible that diet can affect 20E levels and thus regulate stem cell maintenance. In addition to diet, stress can also affect 20E levels, as is the case in *Drosophila virilis*, where 20E levels increase significantly under high temperature stress (Rauschenbach et al., 2000). A similar effect has been found in mammals, where the steroid hormone cortisol is released in response to psychological stressors (Burke et al., 2005; McGaugh, 2004). Finally, 20E levels are also influenced by mating. In *Anopheles gambiae*, males transfer 20E to blood-fed females during copulation, which is important for egg production (Baldini et al., 2013). In female *Drosophila*, whole body ecdysteroid levels also increase after mating (Harshman et al., 1999). Studying the roles of hormonal signaling in mediating stem cell responses to stress and other environmental cues will be an exciting topic for future studies. From our work it is now clear that, as in mammals, steroid signaling plays critical roles in adult stem cell function during both male and female gametogenesis.

## Materials and methods

### *Fly stocks and cultures*

Fly stocks were raised at 25 °C on standard molasses/yeast medium unless otherwise indicated. The following fly stocks were used: *c587-Gal4* (Kai and Spradling, 2003), *E132-Gal4* (from H. Sun), *w*; *Nurf301*$^2$*/TM6B, Tb* and *w;; Nurf301*$^3$*/TM3, Ser* (from P. Badenhorst), *ftz-f1*$^{ex7}$ *FRT2A* (from C. Dauphin-Villemant), *E75*$^{\Delta 51}$ *FRT80B* (from D. Drummond-Barbosa), and *ecd*$^2$ *FRT2A* (from M. Jindra). Other fly stocks came from the Bloomington *Drosophila* Stock Center (BDSC) or Vienna *Drosophila* RNAi Center (VDRC).

### *Immunofluorescence microscopy*

Testes were dissected, fixed, and stained as described previously (Matunis, 1997). The following antibodies were used: rabbit anti-Vasa (d-260) and goat anti-Vasa (dN-13) (Santa Cruz Biotechnology, 1:400); chicken anti-Vasa (from K. Howard, 1:5000); rabbit anti-GFP (Torrey Pines Biolabs, 1:10,000); chicken anti-GFP (Abcam, 1:10,000); mouse anti-β-Galactosidase (Promega, 1:1000); mouse 1B1 (1:25), mouse anti-Armadillo (N2 7A1; 1:50), mouse anti-EcR

(DDA2.7; 1:50), mouse anti-EcR (Ag10.2; 1:50), and mouse anti-Broad-core (25E9.D7; 1:50) (Developmental Studies Hybridoma Bank at the University of Iowa); rabbit anti-ZFH1 (from R. Lehmann, 1:5000); guinea pig anti-ZFH1 (from J. Skeath; 1:1000); guinea pig anti-Tj (from D. Godt, 1:4000); and mouse anti-USP (from D. Montell, 1:20) (Christianson et al., 1992). Alexa fluor-conjugated secondary IgG (H+L) antibodies were diluted at 1:200 for 568 and 633 conjugates and 1:400 for 488 conjugates. Secondary antisera were: goat anti-rat 488, goat anti-rabbit 488 and 568, goat anti-mouse 488, 568 and 633, goat anti-chicken 488 and 568, and goat anti guinea-pig 568 and 633 (Molecular Probes/Invitrogen). DNA was stained with 4,6-diamidino-2-phenylindole (DAPI; Sigma) at 1 mg/ml. Fixed testes were mounted in Vectashield (Vector Labs) for imaging.

### *Analysis of confocal images*

Confocal images were obtained with a Zeiss LSM 5 Pa or a Zeiss LSM 510 Meta microscope and were collected as serial confocal sections at similar detection settings unless otherwise noted. Images were analyzed using the Zeiss LSM Image Browser software or Zen 2009 Light edition software. GSCs were scored as Vasa-positive cells (with a spherical fusome where specified) making contact with the hub. CySCs were scored as Zfh1-positive cells (Leatherman and Dinardo, 2008), with medium to strong staining according to the rainbow indicator in the Zeiss Pascal software. All graphs were created using Prism 5 (GraphPad Software, Inc.). Statistical analysis of stem cell number was performed using Prism 5. Student's *T*-test was used to compare two populations, and unpaired ANOVA analysis was used to compare three or more populations.

### *20E feeding experiment*

20E (Sigma-Aldrich) was dissolved in 10% ethanol to prepare a 25 mM stock solution.

#### *To visualize reporter activity*

Adult males with the genotype *hs-EcR-LBD-GAL4; UAS-stinger* (or *UAS-lacZ*) or *hs-usp-LBD-GAL4*; *UAS-stinger* (or *UAS-lacZ*) were heat shocked 3 × 30 min at 37 °C and then placed in vials containing normal food covered with a piece of filter paper soaked with 100–150 µl of 1 mM 20E (diluted in apple juice) plus green food coloring (McCormick, 1:50). A small hole was cut out of the filter paper to give the flies access to the normal food below. After 1 day, flies with green guts (indicating that they had ingested the 20E) were dissected and stained with GFP or lacZ antibody.

#### *To use reporters as dominant negative constructs*

Adult males with the genotype *hs-EcR-LBD-GAL4*; *UAS-stinger*, or *hs-usp-LBD-GAL4*; *UAS-stinger*, or *hs-ECR-LBD-Gal4*, or *hs-usp-LBD-Gal4* were heat shocked twice everyday for 30 min each time (once in the early morning and once at night) for 4–5 days. For 20E rescue experiments, flies were placed in vials containing 20E (as described above) after each heat shock and dissected one day after the last heat shock. Flies were fed an equivalent concentration of ethanol dissolved in apple juice as a control.

### *Loss-of-function experiments*

To assay the effect of loss of *ecd* on stem cell maintenance, 0–5 day old $ecd^1$ males raised at 18 °C were shifted to non-permissive temperature (29 °C) for 7 days, and then testes were dissected and analyzed. *y w* males were processed in parallel as controls. To assay whether 20E feeding can rescue the $ecd^1$ testis phenotype, we fed flies with 0.1 mM or 1 mM 20E using the method described for the 20E feeding experiment. Flies were fed an equivalent concentration of ethanol dissolved in apple juice as a control.

To assay the effect of loss of *EcR* on stem cell maintenance in adult testes, $EcR^{M554fs}$/*SM6b* (null allele) and $EcR^{A483T}$/*SM6b* (temperature sensitive allele) flies were crossed at permissive temperature (18 °C) and shifted to non-permissive temperature (31 °C) for 7 days, and testes were then dissected and analyzed. Heterozygous sibling males were processed in parallel as controls.

### *Temperature sensitive EcR rescue experiment*

*UAS-EcR.A*, *UAS-EcR.B1*, and *UAS-EcR.B2* constructs were driven by *c587-Gal4* (cyst lineage) or *hh-Gal4* (BDSC 45546; hub cells) in the temperature sensitive *EcR* mutant background ($EcR^{M554fs}$/$EcR^{A483T}$). *UAS-GFP-nls* was used as a control. Flies were grown at 18 °C and transferred to 31 °C as adults to induce expression of the UAS constructs.

### *RNAi and dominant negative (DN) knockdown experiments*

The following RNAi or DN constructs were used for cell type-specific knockdown of ecdysone pathway components:

| Gene | Genotype | Stock number |
|---|---|---|
| EcR | *UAS-EcR-RNAi* | VDRC 37058 |
| | *UAS-EcR-RNAi* | BDSC 9726 |
| | *UAS-EcR.B1-ΔC655.F645A* | BDSC 6869 |
| | *UAS-EcR*.B1-*ΔC655.W650A* | BDSC 6872 |
| | *UAS-EcR.A.F645A* | BDSC 9450 |
| | *UAS-EcR.A.W650A* | BDSC 9451 |
| | *UAS-EcR.B2.F645A* | BDSC 9450 |
| USP | *UAS-USP-RNAi* | VDRC 16893 |
| | *UAS-USP-RNAi* | BDSC 27258 |
| E75 | *UAS-E75-RNAi* | VDRC *44851* |
| ftz-f1 | *UAS-ftz-f1-RNAi* | VDRC 108995 |
| | *UAS-ftz-f1-RNAi* | BDSC *27659* |
| br | *UAS-br-RNAi* | BDSC 27272 |

Male flies carrying these constructs were crossed to females with the genotype *c587-Gal4; tubGAL80$^{ts}$* (cyst lineage) or *E132-Gal; tubGAL80$^{ts}$* (hub cells) at 18 °C. Males were shifted to 29 °C upon eclosion and dissected after 1–5 days. *UAS-GFP RNAi* (BDSC 9330) was used as a control for RNAi experiments and *UAS-GFP* (BDSC 4776) as a control for DN experiments. Flies carrying UAS constructs alone, without a driver, were processed in parallel to check for leakiness of each UAS construct. To look for genetic interaction between ecdysone signaling and NURF, we expressed *UAS-EcR-RNAi* (BDSC 37058) in the CySC lineage in a $Nurf301^3$ or $Nurf301^2$ heterozygous background. *UAS-GFP-RNAi* was used as a control for this experiment.

### *Mosaic analysis*

Negatively marked clones were induced using the FLP, FRT-mediated mitotic recombination technique (Xu and Rubin, 1993) in flies of the genotype: *y w, P[hs-FLP]/Y; P[Ubi-GFP.nls] P[w$^+$ FRT]2A /ftz-f1$^{ex7}$ P[w$^+$ FRT]2A* or *y w, P[hs-FLP]/Y; P[Ubi-GFP.nls] P[w$^+$ FRT] 2A /ecd$^2$ P[w$^+$ FRT]2A* or *y w, P[hs-FLP]/Y; P[Ubi-GFP] P[neoFRT]80B/ E75$^{Δ51}$ P[neoFRT]80B ry$^{506}$*. Control clones were induced in *y w, P[hs-FLP]/Y; P[Ubi-GFP.nls] P[w$^+$ FRT]2A/P[w$^+$ FRT]2A* or *P[hs-FLP]/Y; P[Ubi-GFP] P[neoFRT]80B/P[neoFRT]80B ry$^{506}$*. GSC clones were identified as cells that were Zfh1-negative, GFP-negative, and making broad contact with the hub. CySC clones were identified

as cells that were Zfh1-positive, GFP-negative, and within 2 cell diameters of the hub.

Positively marked clones were induced using the mosaic analysis with a repressible cell marker (MARCM) technique (Lee and Luo, 1999) in flies of the genotype *y w, P[hs-FLP], P[tub-Gal4] P[UAS-CD8-GFP]; P[tub-Gal80] P[w+ FRT]2A/ftz-f1$^{ex7}$ P[w+ FRT]2A*. Control clones were induced in *y w, P[hs-FLP], P[tub-Gal4] P[UAS-CD8-GFP]; P[tub-Gal80] P[w$^+$ FRT]2A/P[w$^+$ FRT]2A* (Wang and Struhl, 2004) (a gift from G. Struhl). CySC clones were identified as cells that were Zfh-1 positive, GFP-positive, and within 2 cell diameters of the hub.

To induce clones, 0–5 day old males were heat shocked for 3 × 30 min at 37 °C separated by 30-minute intervals at 25 °C. Flies were kept at 25 °C for 2, 4, 8, or 10 days after clone induction (ACI) before dissection.

### *Apoptosis detection*

Cells undergoing apoptosis were detected by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL; Chemicon International) as described (Sheng et al., 2009). TUNEL-positive stem cells and early daughters were identified by the position of their nuclei (within two cell diameters of the hub). TUNEL-positive spermatogonia were identified as spots with a diameter greater than 5 μm and located more than two cell diameters from the hub.

## Author contributions

Y.L., Q.M., C.C. and E.M. designed the experiments; Y.L., Q.M., and C.C. performed the experiments; Y.L. and Q.M. analyzed the data; Y.L. and E.M wrote the manuscript.

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2014.07.016.

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