Diet controls *Drosophila* follicle stem cell proliferation via Hedgehog sequestration and release

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A healthy diet improves adult stem cell function and delays diseases such as cancer, heart disease, and neurodegeneration. Defining molecular mechanisms by which nutrients dictate stem cell behavior is a key step toward understanding the role of diet in tissue homeostasis. In this paper, we elucidate the mechanism by which dietary cholesterol controls epithelial follicle stem cell (FSC) proliferation in the fly ovary. In nutrient-restricted flies, the transmembrane protein Boi sequesters Hedgehog (Hh) ligand at the surface of Hh-producing cells within the ovary, limiting FSC proliferation. Upon feeding, dietary cholesterol stimulates S6 kinase–mediated phosphorylation of the Boi cytoplasmic domain, triggering Hh release and FSC proliferation. This mechanism enables a rapid, tissue-specific response to nutritional changes, tailoring stem cell divisions and egg production to environmental conditions sufficient for progeny survival. If conserved in other systems, this mechanism will likely have important implications for studies on molecular control of stem cell function, in which the benefits of low calorie and low cholesterol diets are beginning to emerge.

Introduction

The long-term survival and function of stem cells depend on spatial cues, secreted signals, and structural support generated by the local stem cell microenvironment, or niche (Morrison and Spradling, 2008). Tremendous progress has been made in identifying the niche-generated factors necessary for stem cell regulation and how these factors interact with proteins expressed within the stem cells themselves. In contrast, very little is known about the mechanisms that control stem cell responses to systemic changes within an organism. For example, stem cells proliferate in response to extrinsic factors such as feeding, but the mechanisms that relay systemic nutritional changes to the local stem cell niche have not been well defined.

In *Drosophila melanogaster*, proliferation rates of two ovarian stem cell populations, germline stem cells (GSCs) and epithelial follicle stem cells (FSCs), are controlled by nutritional signals (Drummond-Barbosa and Spradling, 2001). GSCs divide asymmetrically to self-renew and produce a differentiating daughter cell that generates a 16-cell germline cyst, including one cell that is fated to become the oocyte (Fig. 1 A). Developing cysts are enveloped by follicular epithelial cells that are derived from FSCs, resulting in the formation of a follicle cell–germ cell unit called an egg chamber (Fig. 1 A; King, 1970; Spradling, 1993). Under conditions in which flies are fed only simple sugars, GSC and FSC proliferation is arrested to ensure that eggs are not produced when the environment lacks sufficient nutrients to support normal progeny development (Drummond-Barbosa and Spradling, 2001). The starvation response is rapid, with cessation of egg production within 24 h of switching flies to nutrient-restricted food. This effect is reversible, as subsequent feeding of nutrient-restricted flies with rich food activates GSC and FSC proliferation, and normal numbers of eggs are produced within 36–48 h (Drummond-Barbosa and Spradling, 2001). Initiation of egg laying after a period of nutrient deprivation depends on the insulin signaling pathway, which promotes GSC proliferation (LaFever and Drummond-Barbosa, 2005; Hsu et al., 2008; Hsu and Drummond-Barbosa, 2009b, 2011). In contrast, the nutrient-dependent mechanisms that activate FSC proliferation have not been identified.

When abundant nutrients are available, FSC proliferation is controlled through a convergence of Hedgehog (Hh), TGF-β, and Wnt family signals produced by the anterior-most cells within the ovary (apical cells) and Janus kinase–signal transducer

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Abbreviations used in this paper: FC-NA, follicle cell nuclear antigen; FSC, follicle stem cell; GSC, germline stem cell; Hh, Hedgehog; InR, insulin receptor; S6K, S6 kinase; UAS, upstream activation sequence; WT, wild type.

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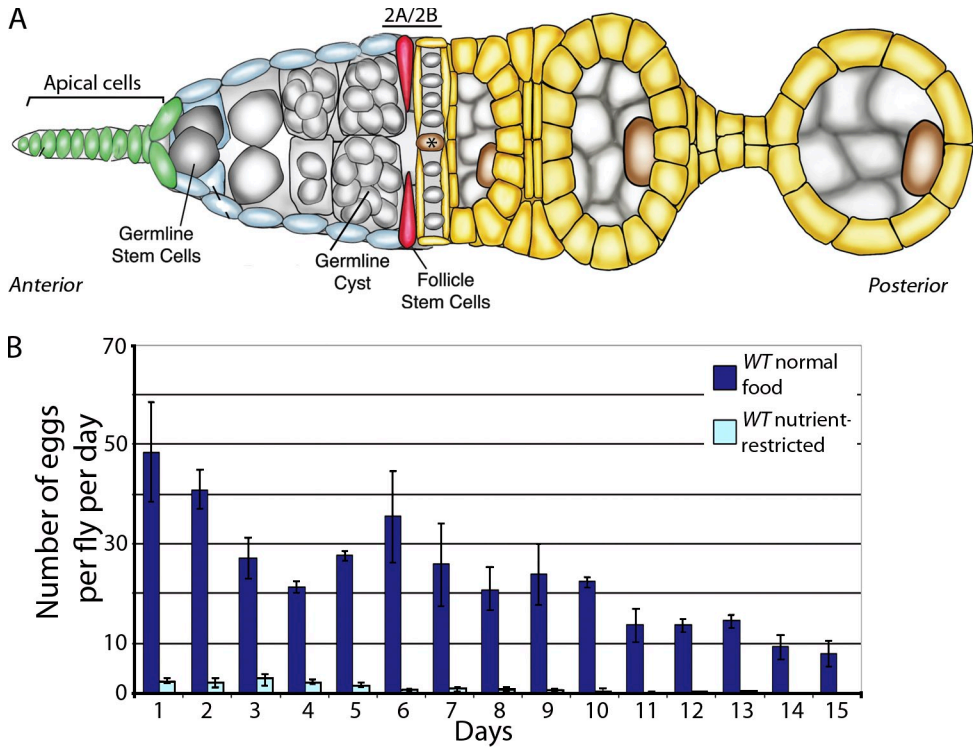

Figure 1. **Nutrient restriction inhibits egg production in flies.** (A) Schematic of early oogenesis. GSCs (gray) contact a cellular niche composed of apical cells (green) found at the anterior of the germarium. FSCs (red) reside three to five cell diameters posterior to apical cells and generate daughter cells (yellow) that form an epithelial layer around 16-cell germline cysts (gray), producing follicles called egg chambers that generate mature eggs in 7 d. The asterisk indicates flattened germline cyst at the region 2A/2B border. (B) Eggs laid/fly/day were scored daily for nutrient-restricted or fed *WT* flies. Error bars represent standard deviations.

and activator of transcription signals induced by cells located to the posterior of FSCs (Fig. 1 A; Forbes et al., 1996a,b; Zhang and Kalderon, 2000, 2001; Song and Xie, 2003; Kirilly et al., 2005; Vied et al., 2012). FSCs express receptors for each of these growth factors and proliferate in response to the presence of ligand in the local niche. The FSC proliferation response is extremely sensitive to the levels of growth factor available. Increased levels of ligand or receptor activity result in excessive FSC proliferation and the accumulation of follicle cells in long cellular stalks between egg chambers. Conversely, too little signaling prevents sufficient FSC proliferation and leads to the generation of egg chambers with gaps in the epithelium, loss of stalk cells, and inappropriate packing of germline cysts (Forbes et al., 1996a,b; Zhang and Kalderon, 2000, 2001; Song and Xie, 2003; Kirilly et al., 2005; Vied et al., 2012).

To maintain the precise rates of FSC proliferation necessary for normal egg chamber development, growth factor levels are tightly regulated through control of ligand production, secretion, and delivery (King et al., 2001; Kirilly et al., 2005; López-Onieva et al., 2008; Guo and Wang, 2009; Hayashi et al., 2009; Szakmary et al., 2009; Liu et al., 2010). Recently, we identified an additional mechanism for regulation of Hh levels in the FSC niche. The transmembrane protein Boi is expressed on the surface of apical cells where it binds directly to Hh, sequestering it away from the FSC niche. In *boi* mutants, Hh is released from apical cells and accumulates near FSCs, where it promotes proliferation (Hartman et al., 2010). Our results indicate that the primary function of Boi in FSC proliferation control is to limit access of Hh ligand to FSCs, thus defining growth factor sequestration as an important mechanism for regulating stem cell proliferation (Hartman et al., 2010). Moreover, these observations suggest that FSC proliferation in wild-type (WT) ovaries may be controlled by triggered release of Hh in response to changes in signals that influence egg production. Here, we demonstrate that Hh sequestration and release are controlled by diet and define the signaling pathway that functions within apical cells to promote Hh release and FSC proliferation control.

Results

To test whether Hh sequestration and release are controlled by nutritional changes, young adult WT females were raised on normal food and then transferred to "nutrient-restricted" conditions consisting only of water and simple sugars (Drummond-Barbosa and Spradling, 2001). Flies can survive on this diet for up to 75 d (mean life span: 30.5 d [restricted] and 40.5 d [fed]; Fig. S1; Hassett, 1948), but they lack essential nutrients, including amino acids, lipids, and vitamins that are necessary for egg production (Fig. 1 B; Drummond-Barbosa and Spradling, 2001). Stem cell proliferation and egg production are stimulated in nutrient-restricted female flies by refeeding the flies yeast, which supplements a sugar-only diet with additional essential nutrients (Drummond-Barbosa and Spradling, 2001). In nutrient-restricted flies expressing Hh-GFP under control of an apical cell–specific Gal4 transcriptional activator (*Hh-GFP/bab-Gal4*; Fig. S2, A and B; Cabrera et al., 2002; Hartman et al., 2010),

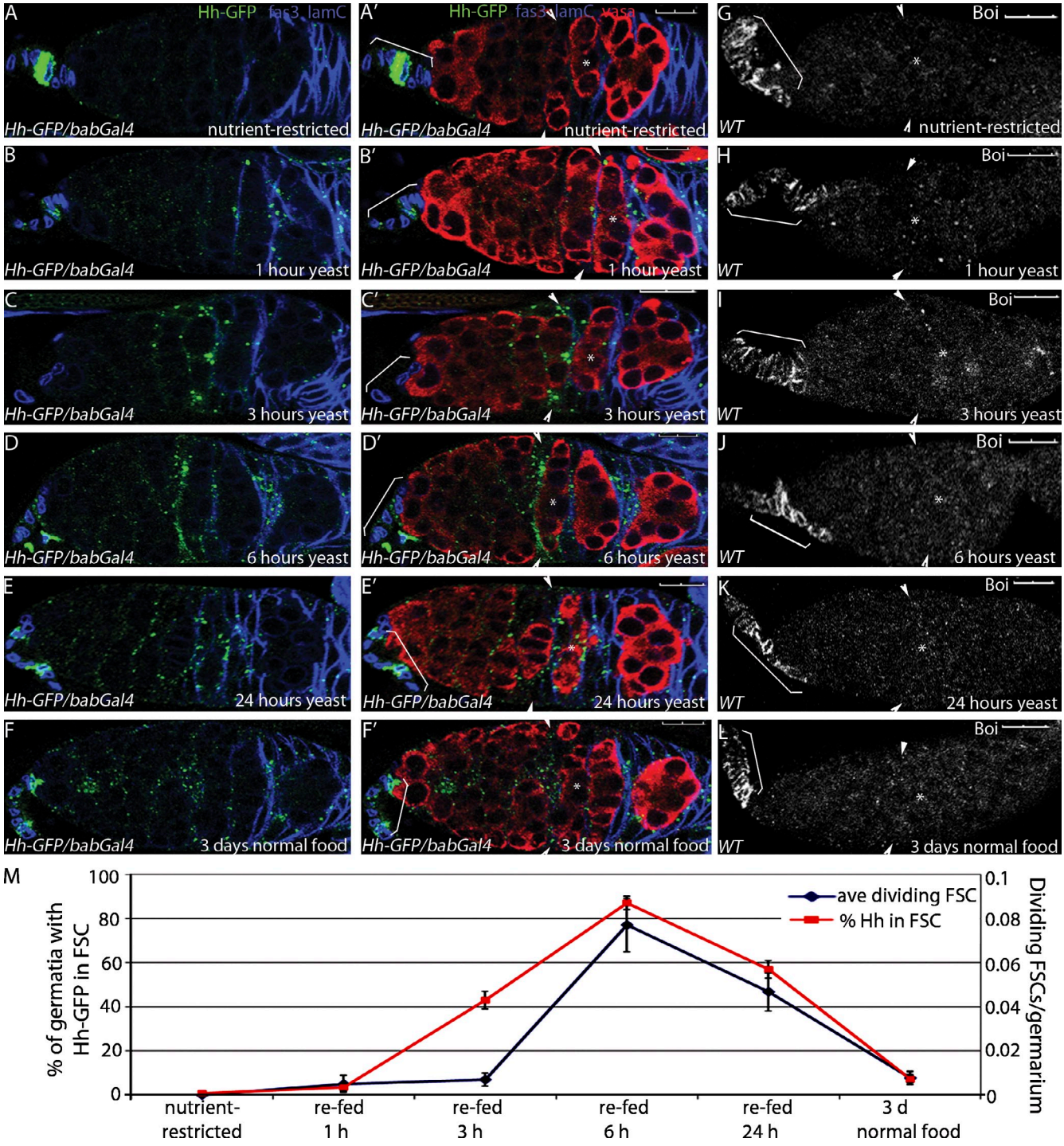

Figure 2. **Refeeding nutrient-restricted flies stimulates Hh release and FSC proliferation.** (A–G) Nutrient-restricted WT flies expressing Hh-GFP in the apical cells (*Hh-GFP/bab-Gal4*) were refed yeast for the times indicated. (A–F) Follicle cells (Fas3) and apical cells (Lamin C [lamC]) are both labeled in blue to enable mapping of Hh localization. Hh-GFP is also shown. (A′–F′) Same images as A–F with germ cells shown (Vasa). (G–L) Boi localization in apical cells is indicated with brackets in nutrient-restricted (G) and refed (H–L) flies. (A–L) Asterisks mark the flattened germline cyst at the region 2A/2B border. Arrowheads indicate FSCs. Brackets indicate apical cells. Bars, 10 µM. (M) Mean numbers of dividing FSCs and Hh-GFP localization in the FSCs are shown. Error bars represent SEs.

Hh-GFP localized primarily to the surface of apical cells (Fig. 2 A) and was rarely seen in other cells within the germarium. By 1 h after feeding, most of the Hh-GFP was released from apical cells (Fig. 2 B). Hh-GFP concentrated in somatic cells in the center of the germarium by 3 h after feeding and peaked by 6 h after feeding (Fig. 2, C and D). These cells exhibit the hallmarks of FSCs, including their location on the surface of the germarium immediately anterior to the first flattened germline cyst at the region 2A/2B border, a characteristic triangular morphology, and low expression of the follicle cell marker Fas3 (Fig. 1 A; Margolis and Spradling, 1995; Zhang and Kalderon, 2001; Nystul and Spradling, 2007, 2010).

Moreover, FSCs are known to be particularly responsive to Hh signaling (Forbes et al., 1996a,b; Zhang and Kalderon, 2000, 2001), supporting the idea that Hh accumulates predominantly within FSCs after release from apical cells. At all time points examined, Boi was expressed on the surface of apical cells, suggesting that the mechanism of Hh release is not caused by loss of Boi from the plasma membrane (Fig. 2, G–L). Production of new Hh-GFP was not observed until 6 h after refeeding (Fig. 2 D), indicating that feeding triggered release of Hh molecules bound to Boi on the surface of apical cells rather than promoting Hh production or secretion. 3 d after refeeding, Hh-GFP localized primarily to the surface of apical cells (Fig. 2 F). A similar time course of Hh release was observed when an antibody that detects endogenous Hh protein was applied to ovaries isolated from nutrient-restricted or refed flies (Fig. S3, A–D). Thus, Hh protein is released from the producing cells in response to nutrient stimulation.

FSCs proliferate in response to Hh (Forbes et al., 1996a; Zhang and Kalderon, 2001), suggesting that nutrient-stimulated Hh accumulation in FSCs might mediate FSC proliferation upon feeding. To measure proliferation, FSCs were identified by nuclear markers that are expressed at higher levels in FSCs and their progeny relative to other cells in the germarium (follicle cell nuclear antigens [FC-NAs]; Fig. S4; König et al., 2011). In addition to marking FSC location and nuclear morphology, high nuclear marker expression correlated precisely with marked FSC clones generated by mitotic recombination using an FSC and follicle cell–specific Gal4 transcriptional activator and upstream activation sequence (UAS)-GFP (*109-30-Gal4*; Figs. S2, C and D; and S4; Hartman et al., 2010). This suggests that nuclear markers can be used to accurately label FSCs and, in contrast to lineage tracing by mitotic recombination, allow the scoring of proliferation in all FSCs of the germarium upon feeding. Germaria also were immunostained with Fas3 to label differentiating follicle cells and anti–phosphohistone-H3, a mitotic mark that is commonly used to identify dividing cells in the germarium (Fig. S4). The time course of FSC proliferation precisely tracked with accumulation of Hh-GFP in FSCs, with increased FSC proliferation observed by 1 h after feeding and peak numbers of dividing FSCs at 6 h (Fig. 2 M). Similar differences in FSC proliferation in nutrient-restricted versus fed flies were observed when germaria were labeled with BrdU (unpublished data; O'Reilly et al., 2008). These results support a model in which feeding triggers increased Hh levels in FSCs to initiate follicle cell production after a nutrient restriction–induced arrest.

Insulin is a key regulator of proliferation of multiple stem cell populations, including GSCs (LaFever and Drummond-Barbosa, 2005; Hsu and Drummond-Barbosa, 2009a; Mairet-Coello et al., 2009; Chell and Brand, 2010; Mathur et al., 2010; McLeod et al., 2010; Michaelson et al., 2010; Sousa-Nunes et al., 2010; O'Brien et al., 2011). However, loss of insulin receptor (InR) expression in FSCs does not affect proliferation (LaFever and Drummond-Barbosa, 2005), and insulin stimulation of germaria cultured ex vivo increases GSC proliferation with no effect on FSC proliferation (unpublished data; Morris and Spradling, 2011). FSC proliferation increased dramatically in females fed complete yeast, indicating that a critical nutrient is present in yeast. In contrast, only a modest response was observed in flies fed yeast extract (Fig. 3 A and Table 1; Horner et al., 2009; Bujold et al., 2010), a rich source of soluble components of yeast, including vitamins, minerals, and the complex sugars and amino acids that are known to stimulate insulin signaling in flies (Géminard et al., 2009; Sousa-Nunes et al., 2010; Musselman et al., 2011). Moreover, reduced expression of the InR in apical cells (*InR RNAI/bab-Gal4*) had no effect on feeding-stimulated Hh release, FSC proliferation, or GSC proliferation (Fig. S5, A–D). In contrast, reduced InR expression in apical cells suppressed proliferation of GSCs and FSCs in well-fed flies (Fig. S5, E and F). These results suggest that insulin is not the primary signaling pathway that mediates the feeding response of nutrient-restricted flies but is essential for maintenance of stem cell proliferation under steady-state conditions. Importantly, these observations suggest that an insulin-independent, hydrophobic component of yeast must act as the primary trigger for FSC proliferation.

Drosophila lack the ability to synthesize cholesterol and must obtain it from the diet (Trager, 1947; Sang, 1956), suggesting it might be a key nutrient for FSC proliferation control. Consistent with this, FSC proliferation was restored in nutrient-restricted flies fed yeast extract supplemented with 0.2 mg/g cholesterol (Fig. 3 A and Table 1). Restored proliferation coincided with Hh release from apical cells and accumulation in FSCs by 6 h after feeding (Figs. 3, B and C; and S3, G–I) in a manner that is indistinguishable from that seen upon feeding flies complete yeast (Figs. 2 D and 3 B). Flies were unable to survive ingestion of cholesterol dissolved in ethanol and could not digest cholesterol in solid form or incorporated into liposomes (unpublished data). These results suggest that dietary cholesterol consumed in the context of other components of a normal diet stimulates Hh release from apical cells to drive FSC proliferation.

Cholesterol absorption and homeostasis in flies are controlled by DHR96, a cholesterol-binding nuclear hormone receptor expressed in the midgut (Horner et al., 2009; Bujold et al., 2010; Sieber and Thummel, 2012). Under nutrient restriction conditions, DHR96 mutants cannot modulate systemic cholesterol levels, resulting in larval lethality (Horner et al., 2009; Bujold et al., 2010). DHR96 is expressed at high levels in the larval midgut (FlyAtlas; King-Jones et al., 2006; Chintapalli et al., 2007), consistent with its requirement in that tissue for function. DHR96 also is expressed at high levels in the adult ovary (FlyAtlas; unpublished data; Chintapalli et al., 2007), suggesting that cholesterol levels might be sensed directly by the ovary in a manner similar to the midgut. Reducing DHR96 levels in apical cells by expressing *RNAi* under control of two independent apical cell–specific Gal4 drivers (*bab-Gal4* and *109-53-Gal4*) dramatically suppressed FSC proliferation upon refeeding (Figs. 3 D and S2 E and Table 1). This effect was caused primarily by reduced DHR96 in apical cells rather than systemic alterations in cholesterol management, as survival of larvae of this genotype on a low cholesterol diet was not affected (unpublished data), and previous work has shown that *bab*-Gal4 does not induce expression in the cholesterol-absorptive cells of the midgut (Cabrera et al., 2002; Sieber and Thummel, 2012).

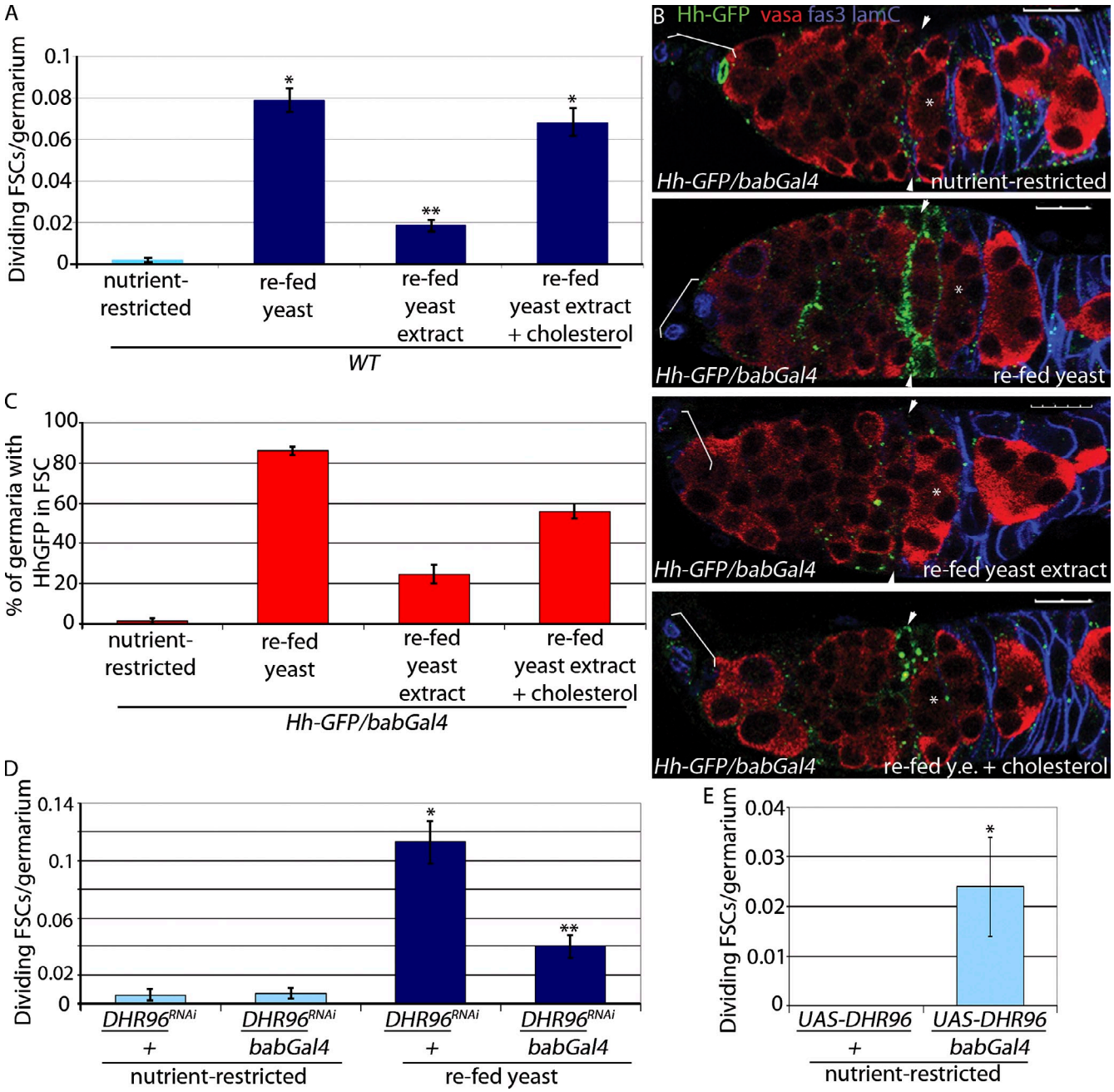

Figure 3. **Cholesterol triggers Hh release.** (A) Nutrient-restricted *WT* flies were refed for 6 h with yeast or yeast extract (y.e.) ± 0.2 mg/g cholesterol or ethanol vehicle control. Mean numbers of dividing FSCs (PH3+) per germarium are shown. *, P < 0.00001 versus nutrient-restricted *WT*. **, P < 0.00001 versus *WT* refed yeast (n = 1,320–2,113; Table 1). (B and C) Nutrient-restricted *WT* flies expressing Hh-GFP in apical cells (*Hh-GFP/bab-Gal4*) were refed for 6 h with yeast or yeast extract ± 0.2 mg/g cholesterol or ethanol vehicle control and stained for Hh-GFP. (B) Follicle cells and apical cells are both labeled in blue (Fas3 and Lamin C [lamC], respectively), and germ cells are labeled red (Vasa). Asterisks indicate flattened germline cyst at the region 2A/2B border. Arrowheads indicate FSCs. Brackets indicate apical cells. Bars, 10 µM. (C) The percentage of germaria with Hh-GFP localized to FSCs was scored (n = 85–195). (D) Nutrient-restricted *DHR96^{RNAi}/bab-Gal4* and *DHR96^{RNAi}/+* flies were refed yeast for 6 h. Mean numbers of dividing FSCs (PH3+) per germarium are shown. *, P < 0.00001 versus nutrient-restricted *DHR96 RNAi/+*. **, P < 0.00001 versus refed *DHR96 RNAi/+* (n = 779–1,194; Table 1). (E) *UAS-DHR96/bab-Gal4* and *UAS-DHR96/+* flies were nutrient restricted for 3 d. Mean numbers of dividing FSCs (PH3+) per germarium are shown. *, P < 0.005 versus nutrient-restricted *UAS-DHR96/+* (n = 184 and 251; Table 1). Error bars represent SEs.

Overexpression of DHR96 in fly larvae promotes survival in starved animals (Sieber and Thummel, 2009), caused either by the ability to scavenge remaining cholesterol molecules in starved flies or the ability to activate downstream signaling in the absence of ligand. Consistent with this, increased DHR96 expression in apical cells promoted FSC proliferation in nutrient-restricted females modestly (Fig. 3 E), supporting the idea that DHR96 activity is sufficient to promote FSC proliferation.

As expected, nutrient-stimulated FSC proliferation depended on Hh signaling. FSCs expressing RNAi targeted against the Hh pathway effector (*109-30-Gal4/smo^{RNAi}*; Fig. S2; Hartman et al., 2010) exhibited significantly diminished proliferation in

Table 1. **Quantification of FSC proliferation**

Genotype	Control genotype	Scoring average		P-value			
		Starve conditions	Refed yeast 6 h	Starve vs. control starve	Starve vs. control refed	Refed vs. control starve	Refed vs. control refed
w1118	w1118	0.0015 (0.001); *n* = 1,948	0.09 (0.006); *n* = 2,113	na	≤0.00001	≤0.00001	na
boi^{e01708}	w1118	0.054 (0.012); *n* = 370	0.13 (0.016); *n* = 437	≤0.00001	≤0.023	≤0.00001	≤0.01
bab-Gal4/+	bab-Gal4/+	0.002 (0.002); *n* = 457	0.088 (0.012); *n* = 522	na	≤0.00001	≤0.00001	na
DHR96 RNAi/+	bab-Gal4/+	0.002 (0.0017); *n* = 825	0.113 (0.011); *n* = 779	≤0.99	≤0.00001	≤0.00001	≤0.15
DHR96 RNAi/ bab-Gal4	bab-Gal4/+	0.005 (0.002); *n* = 1,014	0.039 (0.006); *n* = 1,194	≤0.4	≤0.00001	≤0.00001	≤0.00001
UAS-DHR96/+	bab-Gal4/+	0 (0); *n* = 184	0.102 (0.019); *n* = 264	≤0.56	≤0.00001	≤0.00001	≤0.52
UAS-DHR96/ bab-Gal4	bab-Gal4/+	0.024 (0.01); *n* = 251	0.097 (0.01); *n* = 487	≤0.005	≤0.00001	≤0.00001	≤0.62
Hh RNAi/+	bab-Gal4/+	0 (0); *n* = 429	0.135 (0.016); *n* = 438	≤0.38	≤0.00001	≤0.00001	≤0.02
Hh RNAi/ bab-Gal4	bab-Gal4/+	0.004 (0.004); *n* = 278	0.05 (0.008) *n* = 699	≤0.61	≤0.00001	≤0.00001	≤0.008
boi^{e}; bab-Gal4/+	bab-Gal4/+	0.05 (0.01); *n* = 418	0.099 (0.017); *n* = 314	≤0.00001	≤0.00001	≤0.00001	≤0.59
boi^{e}; DHR96 RNAi/+	bab-Gal4/+	0.051 (0.008); *n* = 800	0.11 (0.018); *n* = 290	≤0.008	≤0.008	≤0.00001	≤0.31
boi^{e}; DHR96 RNAi/ bab-Gal4	bab-Gal4/+	0.038 (0.007); *n* = 710	0.074 (0.011); *n* = 569	≤0.00001	≤0.0002	≤0.00001	≤0.39
S6K RNAi/+	bab-Gal4/+	0.005 (0.002); *n* = 1,037	0.093 (0.01); *n* = 724	≤0.39	≤0.00001	≤0.00001	≤0.76
S6K RNAi/ bab-Gal4	bab-Gal4/+	0.002 (0.002); *n* = 493	0.042 (0.007); *n* = 935	≤0.99	≤0.00001	≤0.00001	≤0.0004
UAS-S6K-TE/+	bab-Gal4/+	0.003 (0.002); *n* = 1,132	na	≤0.72	≤0.00001	na	na
UAS-S6K-WT/ bab-Gal4	bab-Gal4/+	0.005 (0.003); *n* = 595	na	≤0.43	≤0.00001	na	na
UAS-S6K-TE/ bab-Gal4	bab-Gal4/+	0.018 (0.008); *n* = 277	na	≤0.02	<0.0002	na	na
UAS-S6K-STDE/+	bab-Gal4/+	0.003 (0.002); *n* = 975	na	≤0.74	≤0.00001	na	na
UAS-S6K-STDE/ bab-Gal4	bab-Gal4/+	0.016 (0.005); *n* = 708	na	≤0.02	≤0.00001	na	na
S6K RNAi/+; UAS-DHR96/bab-Gal4	bab-Gal4/+	0.006 (0.003); *n* = 518	na	≤0.33	≤0.00001	na	na
S6K RNAi/+; UAS-DHR96/bab-Gal4	UAS-DHR96/bab-Gal4	0.006 (0.003); *n* = 518	na	<0.03	na	na	na
boi^{e}; S6K RNAi/+; +/TM2	boi^{e}; S6KRNAi/+; +/TM2	0.015 (0.006); *n* = 337	na	na	na	na	na
boi^{e}; S6K RNAi/+; bab-Gal4/TM2	boi^{e}; S6KRNAi/+; +/TM2	0.012 (0.008); *n* = 163	na	≤0.789	na	na	na
InR-JF01183/+	InR-JF01183/+	0.001 (0.001); *n* = 830	0.057 (0.009); *n* = 716	na	≤0.00001	≤0.00001	na
InR-JF01183/ bab-Gal4	InR-JF01183/+	0.003 (0.002); *n* = 750	0.061 (0.01); *n* = 539	≤0.36	≤0.00001	≤0.00001	≤0.77
InR-JF01482/+	InR-JF01482/+	0 (0); *n* = 252	0.078 (0.013); *n* = 448	na	≤0.00001	≤0.00001	na
InR-JF01482/ bab-Gal4	InR-JF01482/+	0 (0); *n* = 146	0.091 (0.015); *n* = 364	≤0.999	≤0.0004	≤0.00001	≤0.51
109-53-Gal4/+	109-53-Gal4/+	0.002 (0.002); *n* = 518	0.096 (0.012); *n* = 634	na	≤0.00001	≤0.00001	na
DHR96 RNAi/+	109-53-Gal4/+	0 (0); *n* = 386	0.088 (0.013); *n* = 513	≤0.37	≤0.00001	≤0.00001	≤0.64
DHR96 RNAi/ 109-53-Gal4	109-53-Gal4/+	0.002 (0.002); *n* = 444	0.048 (0.009); *n* = 543	≤0.99	<0.00001	<0.00001	<0.002

Table 1. **Quantification of FSC proliferation** *(Continued)*

Genotype	Control genotype	Scoring average		P-value			
		Starve conditions	**Refed yeast 6 h**	**Starve vs. control starve**	**Starve vs. control refed**	**Refed vs. control starve**	**Refed vs. control refed**
109-30-Gal4/+	109-30-Gal4/+	0.002 (0.002); *n* = 455	0.067 (0.009); *n* = 715	na	≤0.00001	≤0.00001	na
smo RNAi/+	109-30-Gal4/+	0.005 (0.004); *n* = 336	0.086 (0.009); *n* = 924	≤0.37	≤0.00001	≤0.00001	<0.16
smo RNAi/109-30-Gal4	109-30-Gal4/+	0.003 (0.002); *n* = 1,069	0.026 (0.004); *n* = 1,294	≤0.73	≤0.00001	≤0.001	≤0.00001
boi^e; UAS-Boi/bab-Gal4	boi^e; UAS-Boi/bab-Gal4	0 (0); *n* = 314	0.118 (0.016); *n* = 398	na	≤0.00001	≤0.00001	na
boi^e; UAS-BoiΔFN1/+	boi^e; UAS-Boi/bab-Gal4	0.032 (0.01); *n* = 308	0.056 (0.01); *n* = 550	≤0.001	≤0.00001	≤0.00001	<0.0006
boi^e; UAS-BoiΔFN1/bab-Gal4	boi^e; UAS-Boi/bab-Gal4	0.029 (0.01); *n* = 243	0.043 (0.009); *n* = 534	≤0.002	≤0.00001	<0.0002	<0.00001
boi^e; UAS-BoiΔcyto/bab-Gal4	boi^e; UAS-Boi/bab-Gal4	0.002 (0.002); *n* = 409	0.025 (0.007); *n* = 563	≤0.47	≤0.00001	≤0.005	<0.00001
boi^e; UAS-BoiΔcyto-GFP/bab-Gal4	boi^e; UAS-Boi/bab-Gal4	0.003 (0.002); *n* = 621	0.028 (0.007); *n* = 565	≤0.35	≤0.00001	≤0.003	<0.00001
boi^e; UAS-BoiΔcterm/bab-Gal4	boi^e; UAS-Boi/bab-Gal4	0.003 (0.002); *n* = 755	0.014 (0.004); *n* = 725	≤0.30	≤0.00001	≤0.034	<0.00001
boi^e; UAS-BoiΔcterm-GFP/bab-Gal4	boi^e; UAS-Boi/bab-Gal4	0 (0); *n* = 157	0.009 (0.006); *n* = 218	≤0.99	≤0.00001	≤0.096	<0.00001
boi^e; EP-lhog/bab-Gal4	boi^e; UAS-Boi/bab-Gal4	0.005 (0.003); *n* = 753	0.077 (0.012); *n* = 480	≤0.22	≤0.00001	≤0.00001	<0.04
boi^e; UAS-Boi983A/bab-Gal4	boi^e; UAS-Boi/bab-Gal4	0.004 (0.003); *n* = 460	0.022 (0.005); *n* = 962	≤0.28	≤0.00001	≤0.008	≤0.00001
			Refed yeast extract 6 h		**Starve vs. control refed yeast extract**	**Refed yeast extract vs. control starve**	**Refed yeast extract vs. control refed yeast extract**
w1118	w1118	0.002 (0.001); *n* = 1,538	0.019 (0.003); *n* = 1,542	na	≤0.00001	≤0.00001	na
109-30-Gal4/+	109-30-Gal4/+	0.002 (0.002); *n* = 455	0.018 (0.009); *n* = 217	na	<0.024	<0.024	na
smo RNAi/+	109-30-Gal4/+	0.005 (0.004); *n* = 336	0.014 (0.005); *n* = 509	na	≤0.27	≤0.27	na
smo RNAi/109-30-Gal4	109-30-Gal4/+	0.003 (0.002); *n* = 1,069	0.021 (0.009); *n* = 235	≤0.42	≤0.01	≤0.11	≤0.48
			Refed yeast extract with cholesterol 6 h		**Starve vs. control refed cholesterol**	**Refed cholesterol vs. control starve**	**Refed cholesterol vs. control refed cholesterol**
w1118	w1118	0.002 (0.001); *n* = 1,538	0.068 (0.007); *n* = 1,320	na	≤0.00001	≤0.00001	na
109-30-Gal4/+	109-30-Gal4/+	0.002 (0.002); *n* = 455	0.096 (0.014); *n* = 438	na	≤0.00001	≤0.00001	na
smo RNAi/+	109-30-Gal4/+	0.005 (0.004); *n* = 336	0.13 (0.018); *n* = 346	na	≤0.00001	≤0.00001	na
smo RNAi/109-30-Gal4	109-30-Gal4/+	0.003 (0.002); *n* = 1,069	0.023 (0.007); *n* = 514	≤0.42	≤0.00001	≤0.06	≤0.00001
		Refed yeast extract 6 h	**Refed yeast extract with cholesterol 6 h**	**Refed yeast extract vs. control refed yeast extract**	**Refed yeast extract vs. control refed cholesterol**	**Refed cholesterol vs. control refed yeast extract**	**Refed cholesterol vs. control refed cholesterol**
w1118	w1118	0.019 (0.003); *n* = 1,542	0.068 (0.007); *n* = 1,320	na	≤0.00001	≤0.00001	na
109-30-Gal4/+	109-30-Gal4/+	0.018 (0.009); *n* = 217	0.096 (0.014); *n* = 438	na	≤0.0002	≤0.0002	na
smo RNAi/+	109-30-Gal4/+	0.014 (0.005); *n* = 509	0.13 (0.018); *n* = 346	na	≤0.00001	≤0.00001	na
smo RNAi/109-30-Gal4	109-30-Gal4/+	0.021 (0.009); *n* = 235	0.023 (0.007); *n* = 514	≤0.48	≤0.00001	≤0.29	≤0.00001

Number of germarium scored per genotype for each condition is shown (*n* = x). Mean numbers are shown with SE in parentheses. Two-sample Student's *t* test uses for all statistical analysis. Significant differences are achieved at P ≤ 0.05. na, not applicable.

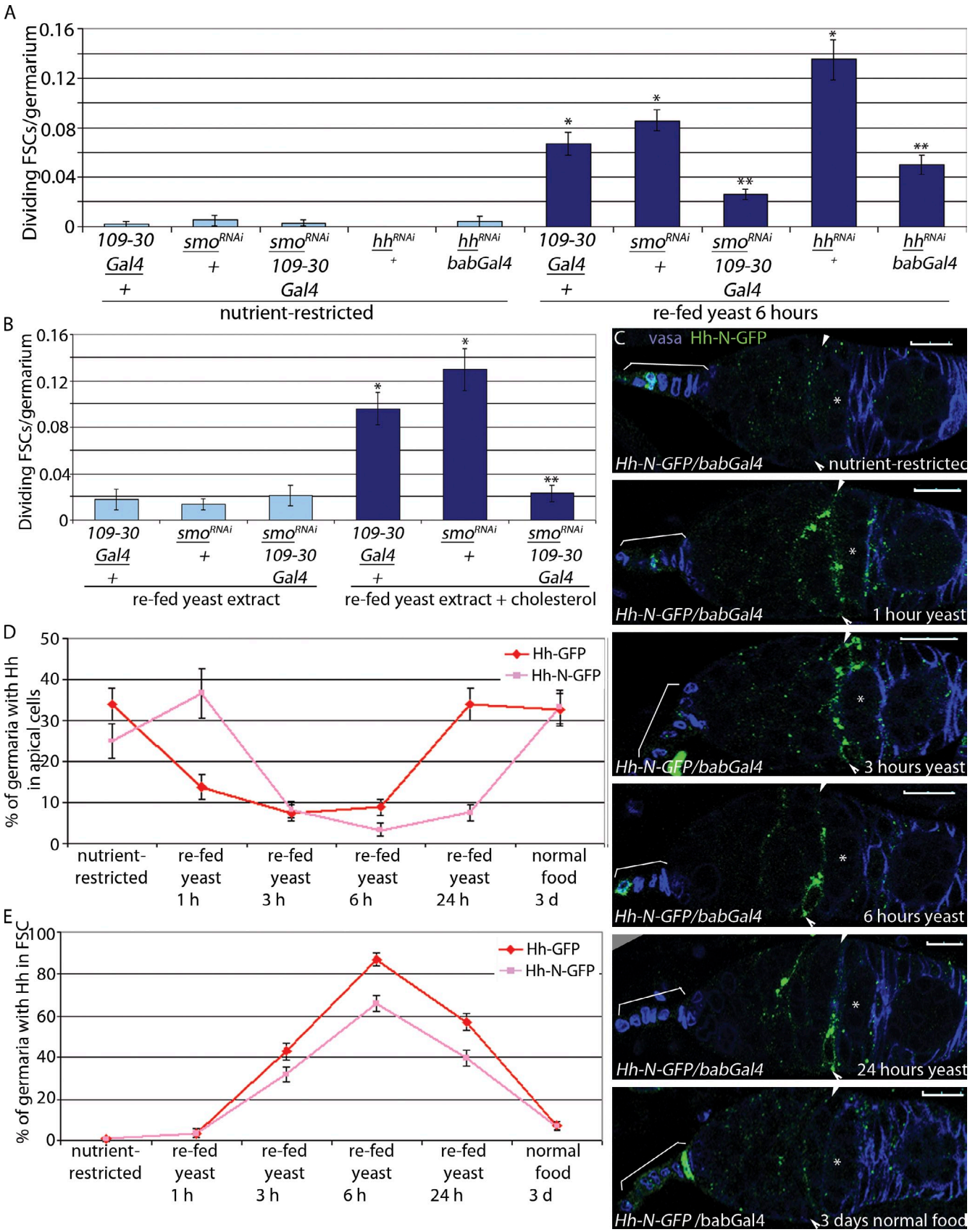

Figure 4. **Cholesterol-mediated Hh release is sufficient for stem cell proliferation.** (A and B) Nutrient-restricted smo^{RNAi}/*109-30-Gal4*, smo^{RNAi}/+, and *109-30-Gal4*/+ flies were refed yeast (A) or yeast extract ± 0.2 mg/g cholesterol (B) for 6 h. Mean numbers of dividing FSCs (PH3+) per germarium are shown. *, P < 0.00001 versus nutrient-restricted *109-30-Gal4*/+. **, P < 0.00001 versus *smo RNAi*/+ refed yeast (n = 366–1,294 [A] and n = 217–514 [B]; Table 1). (A) Nutrient-restricted hh^{RNAi}/*bab-Gal4* and hh^{RNAi}/+ flies were refed yeast paste for 6 h. *, P < 0.00001 versus nutrient-restricted

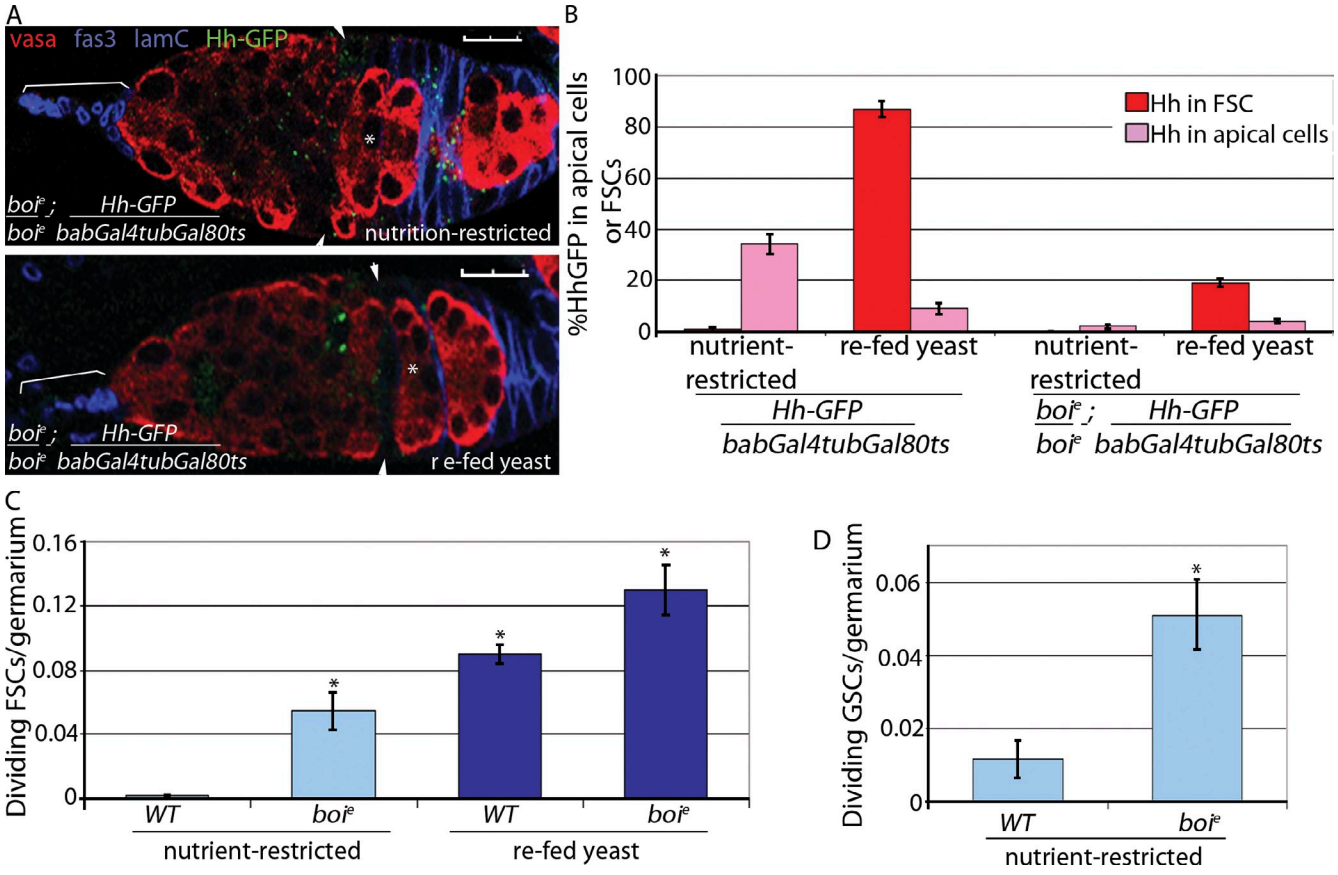

Figure 5. **FSCs proliferate in nutrient-restricted *boi* mutant flies.** (A and B) *WT* and *boi*^{e} mutant flies expressing Hh-GFP in apical cells (*Hh-GFP/bab-Gal4tubGal80ts* and *boi*^{e}*; Hh-GFP/bab-Gal4tubGal80ts*) were nutrient restricted or refed yeast and stained for Hh-GFP. Follicle cells (Fas3) and apical cells (Lamin C [LamC]) are both labeled in blue to enable mapping of Hh-GFP localization, and germ cells are labeled red (Vasa; A). Asterisks indicate flattened germline cyst at the region 2A/2B border. Arrowheads indicate FSCs. Brackets indicate apical cells. Bars, 10 µM. (B) Percentage of germaria with Hh-GFP localized to apical cells or FSCs was quantified; n = 146–654. (C and D) Nutrient-restricted *WT* and *boi*^{e} mutant flies were refed yeast for 6 h. Mean numbers of dividing FSCs (*, P < 0.00001 vs. nutrient-restricted *WT* [n = 370–2,113; Table 1]; C) or GSCs (*, P < 0.0007 vs. nutrient-restricted *WT* [n = 427 and 527]; D) per germarium are shown. Error bars represent SEs.

response to yeast (Fig. 4 A and Table 1) or to yeast extract plus cholesterol (Fig. 4 B and Table 1), indicating that cholesterol promotes FSC proliferation via activation of Hh signaling within FSCs. RNAi-mediated reduction of Hh in apical cells also significantly suppressed FSC proliferation after feeding (Fig. 4 A), suggesting that apical cells are the primary source of Hh ligand for FSC proliferation control. Active Hh ligand (Hh-N) is generated by cleavage of a precursor form of the protein followed by addition of a cholesterol moiety to the newly generated C terminus (Eaton, 2008), suggesting that cholesterol from the diet may be necessary for generating active, cholesterol-modified Hh-N. However, a recombinant Hh-N that cannot be cholesterol modified (Hh-N–GFP) was sequestered on the surface of apical cells in nutrient-restricted WT flies (*Hh-N–GFP/bab-Gal4*) and released in a manner similar to that observed for cholesterol-modified Hh-GFP (Figs. 2, A–F; and 4, C–E), indicating that cholesterol modification is not necessary for the observed effects. These results support a model in which dietary cholesterol-mediated release of active Hh from Boi promotes FSC proliferation via Smo activation within FSCs.

If Hh is a primary nutrient-responsive signal, Hh signaling should be sufficient to stimulate FSC proliferation, regardless of nutrition status. In *boi* mutant females, Hh is constitutively released from apical cells, but significant accumulation of Hh within FSCs is not observed (Fig. 5, A and B; Hartman et al., 2010). Most likely, Hh ligand is used by FSCs continuously in *boi* mutants rather than accumulating within FSCs after the simultaneous release of many Hh molecules upon feeding nutrient-restricted WT flies (Fig. 2). Consistent with this idea, FSCs in *boi* mutants continued to proliferate in nutrient-restricted flies (Fig. 5 C and Table 1). GSC proliferation also was fivefold higher in *boi* mutants versus WT flies under nutrient restriction conditions (Fig. 5 D), consistent with previous observations that Hh signaling can promote GSC proliferation under

bab-Gal4/+. **, P < 0.00001 versus *hh*^{RNAi}*/+* refed yeast (n = 278–699; Table 1). Error bars represent SEs. (C–E) Active Hh-N–GFP is not modified by cholesterol but is retained in the apical cells in nutrient-restricted flies and released in refed flies. Asterisks indicate flattened germline cyst at the region 2A/2B border. Arrowheads indicate FSCs. Brackets indicate apical cells. Bars, 10 µM. Hh-N–GFP is lost from the apical cells (C and D) and accumulates in FSCs (C and E) at similar levels observed with cholesterol-modified Hh-GFP-C. Error bars represent SEs.

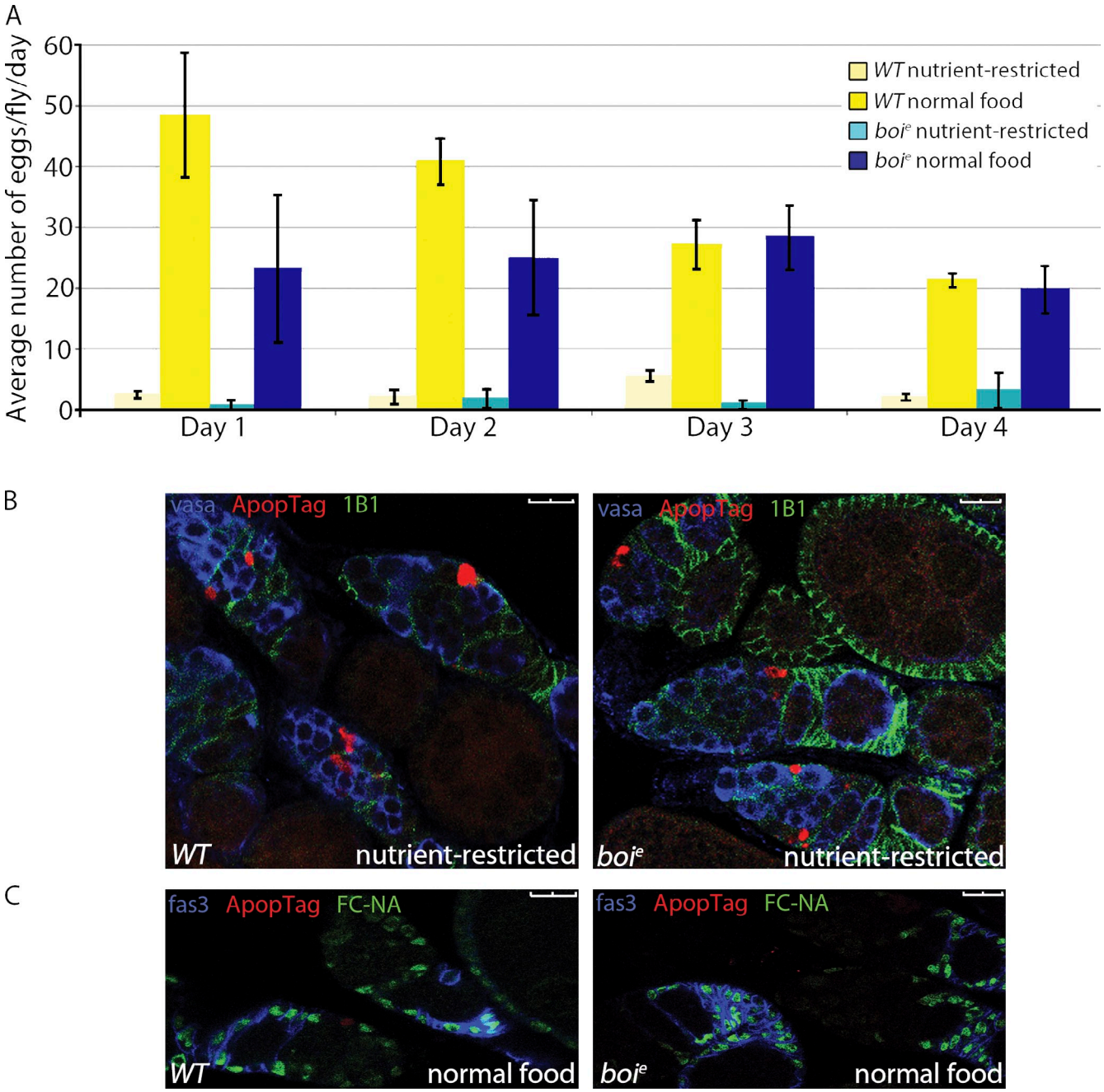

Figure 6. **Nutrient restriction blocks egg production in Boi mutant flies.** (A) *WT* and *boi*^{e} mutant flies were nutrient restricted or fed yeast, and eggs laid per fly were scored daily. Error bars represent standard deviations. (B) Germaria from nutrient-restricted *WT* or *boi*^{e} mutant females were immunostained for follicle cells (1B1), germ cells (Vasa), and apoptosis (ApopTag). (C) *WT* or *boi*^{e} mutant flies on normal food lacked apoptosis (nuclei [FC-NA]), follicle cells (Fas3), and apoptosis (ApopTag). Bars, 10 µM.

conditions in which normal proliferation signals are disrupted (King et al., 2001). Although *boi* mutant females resisted nutrient restriction, feeding with complete yeast further stimulated proliferation (Fig. 5 C). This may be a result of the additional Hh produced 6 h after feeding (Fig. 2 D) or to a second nutrient-dependent mechanism that supplements Hh signals to promote FSC proliferation. Despite constitutive proliferation of GSCs and FSCs in starved *boi* mutants, egg laying was negligible after 1 d because of massive apoptosis of germline cysts in the germarium similar to that seen in nutrient-restricted WT flies (Fig. 6, A and B; Buszczak and Cooley, 2000; Drummond-Barbosa and Spradling, 2001; Pritchett et al., 2009). In contrast, apoptosis of germline cysts was rarely seen in fed WT or *boi* mutants (Fig. 6 C). Thus, Hh release is sufficient for stem cell proliferation, but additional nutritional signals promote germline cyst survival (Terashima and Bownes, 2005; Terashima et al., 2005; Pritchett and McCall, 2012).

Expression of WT Boi or its close relative Ihog in apical cells was sufficient to rescue the FSC proliferation defects in nutrient-restricted *boi* mutants (Fig. 7 A and Table 1). Both

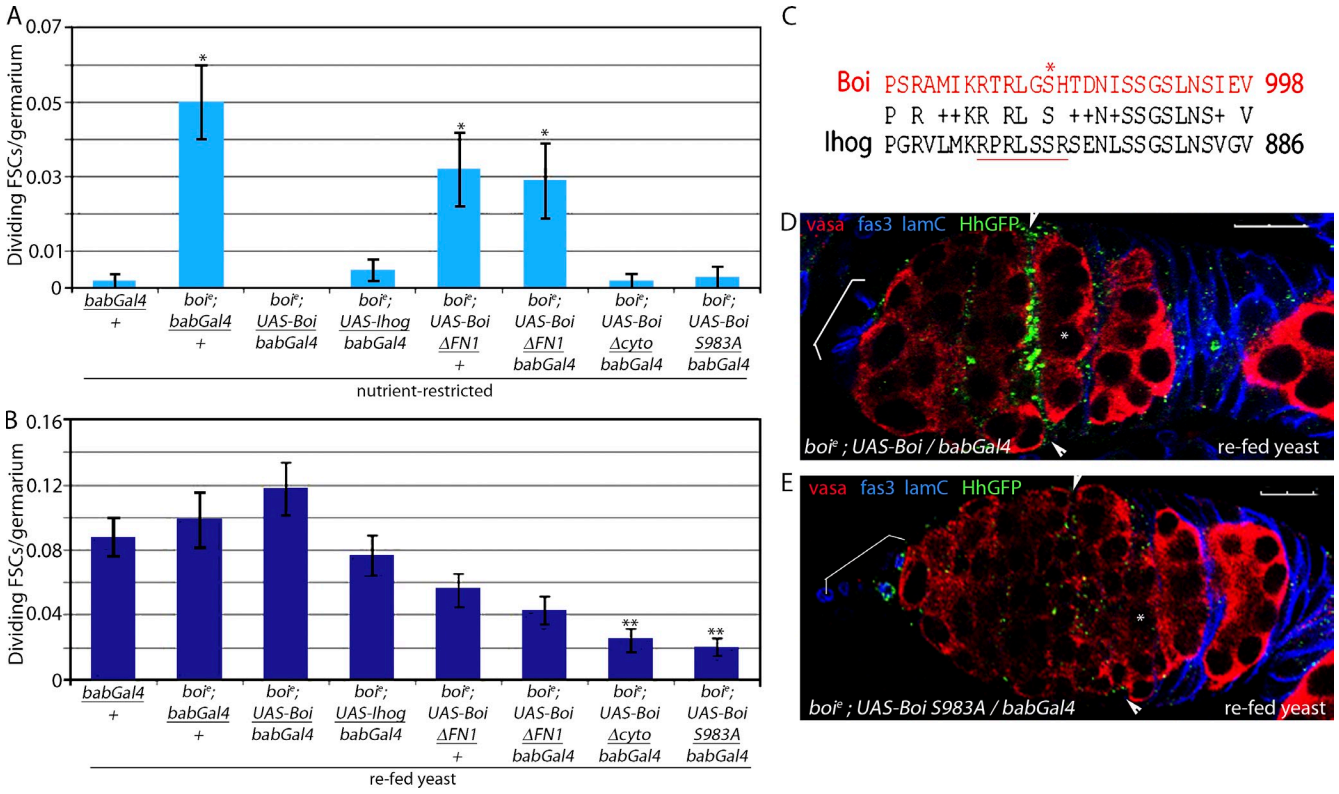

Figure 7. **Hh release is controlled by phosphorylation of a conserved serine in the Boi cytoplasmic domain.** (A and B) *boi*^{e} mutant flies rescued with the indicated form of Boi were nutrient restricted for 3 d (A) and refed yeast for 6 h (B). (A) Forms of Boi with the ability to bind Hh rescued FSC overproliferation in nutrient-restricted *boi*^{e} mutants, consistent with their ability to sequester Hh. *, P < 0.00001 versus nutrient-restricted *bab-Gal4/+* (n = 243–753; Table 1). (B) Forms of Boi lacking key regions of the cytoplasmic domain failed to fully rescue FSC proliferation in fed flies, consistent with a failure to release Hh. **, P < 0.00001 versus *boi*^{e}*; UAS-boi/bab-Gal4* refed yeast (n = 314–962; Table 1). Error bars represent SEs. (C) Conserved region in the cytoplasmic domains of Boi and Ihog. S983 (asterisk) and the S6K consensus site (RxRxxSx, underlined) are indicated. The alignment was performed using NCBI BLAST (Basic Local Alignment Search Tool). Letters that are identical between two sequences are reported. Those that have positive scores in the scoring matrix are displayed with a plus sign. Gaps and nonpositive scores are blank. (D and E) *boi*^{e} mutant flies expressing HhGFP and WT Boi (*boi*^{e}*; HhGFP/+; UAS-Boi/bab-Gal4*) accumulate Hh-GFP in FSCs after refeeding (D), whereas flies expressing Boi^{S983} (*boi*^{e}*; HhGFP/+; UAS-boi*^{S983A}*/bab-Gal4*) do not accumulate HhGFP in FSCs after refeeding (E). Hh-GFP, follicle cells (Fas3), apical cells (Lamin C [lamC]), and germ cells (Vasa) are shown. Asterisks indicate flattened germline cyst at the region 2A/2B border. Arrowheads indicate FSCs. Brackets indicate apical cells. Bars, 10 µM.

proteins have the Hh-binding domains and Patched interaction domains required for initiating Smo-dependent signaling in flies and mammals (Cole and Krauss, 2003; McLellan et al., 2006, 2008; Tenzen et al., 2006; Yao et al., 2006; Beachy et al., 2010; Zheng et al., 2010; Bae et al., 2011; Izzi et al., 2011). However, Hh sequestration in well-fed flies requires only the Hh-binding domain of Boi (Hartman et al., 2010). Consistent with this observation, expression of Boi lacking the Hh-binding domain (Boi$^{\Delta FN1}$) failed to rescue FSC proliferation in nutrient-restricted *boi* mutants (Fig. 7 A and Table 1). A form of Boi that retains the extracellular domain of Boi, including the Hh-binding region, but lacks the cytoplasmic domain (Boi$^{\Delta cyto}$), suppressed FSC proliferation in nutrient-restricted *boi* mutants (Fig. 7 A and Table 1). However, Hh binding was not sufficient to rescue FSC proliferation upon refeeding. Expression of Boi$^{\Delta cyto}$ did not permit stimulation of FSC proliferation to WT levels upon refeeding (Fig. 7 B and Table 1), indicating that the cytoplasmic domain of Boi is necessary for feeding-stimulated Hh release. The capacity of Ihog to rescue all *boi* mutant defects (Fig. 7, A and B; and Table 1) suggested that the triggering mechanism is conserved in Boi and Ihog. A sequence comparison revealed that only a 28–amino acid sequence at the C terminus is conserved between the two proteins (Fig. 7 C). A form of Boi bearing a 28–amino acid C-terminal deletion (Boi$^{\Delta C\text{-term}}$) rescued FSC proliferation defects in nutrient-restricted *boi* mutant flies but failed to rescue feeding-stimulated FSC proliferation, indicating a critical role for the conserved region (Table 1). This sequence includes a serine residue (S983) in Boi known to be phosphorylated in vivo in fly embryos (Zhai et al., 2008), suggesting that S983 phosphorylation might trigger Hh release. Consistent with this model, Hh release and FSC proliferation were suppressed in refed flies expressing only a mutant form of Boi bearing a mutation of S983 to alanine (Boi^{S983A}) under conditions in which WT Boi fully rescued Hh release (Fig. 7, B, D, and E; and Table 1). As expected, Boi^{S983A} was able to rescue Hh sequestration and FSC proliferation defects in nutrient-restricted *boi* mutants (Fig. 7 A and Table 1) because it retains the ability to bind to Hh (Fig. S3, E and F).

S983 matches the established consensus site for S6 kinase (S6K; Flotow and Thomas, 1992). In vitro, S6K robustly phosphorylated the cytoplasmic domain of WT Boi, but no phosphorylation was observed in Boi^{S983A} (Fig. 8 A). S6K-mediated S983 phosphorylation is critical for FSC proliferation control because reduced expression of S6K in apical cells

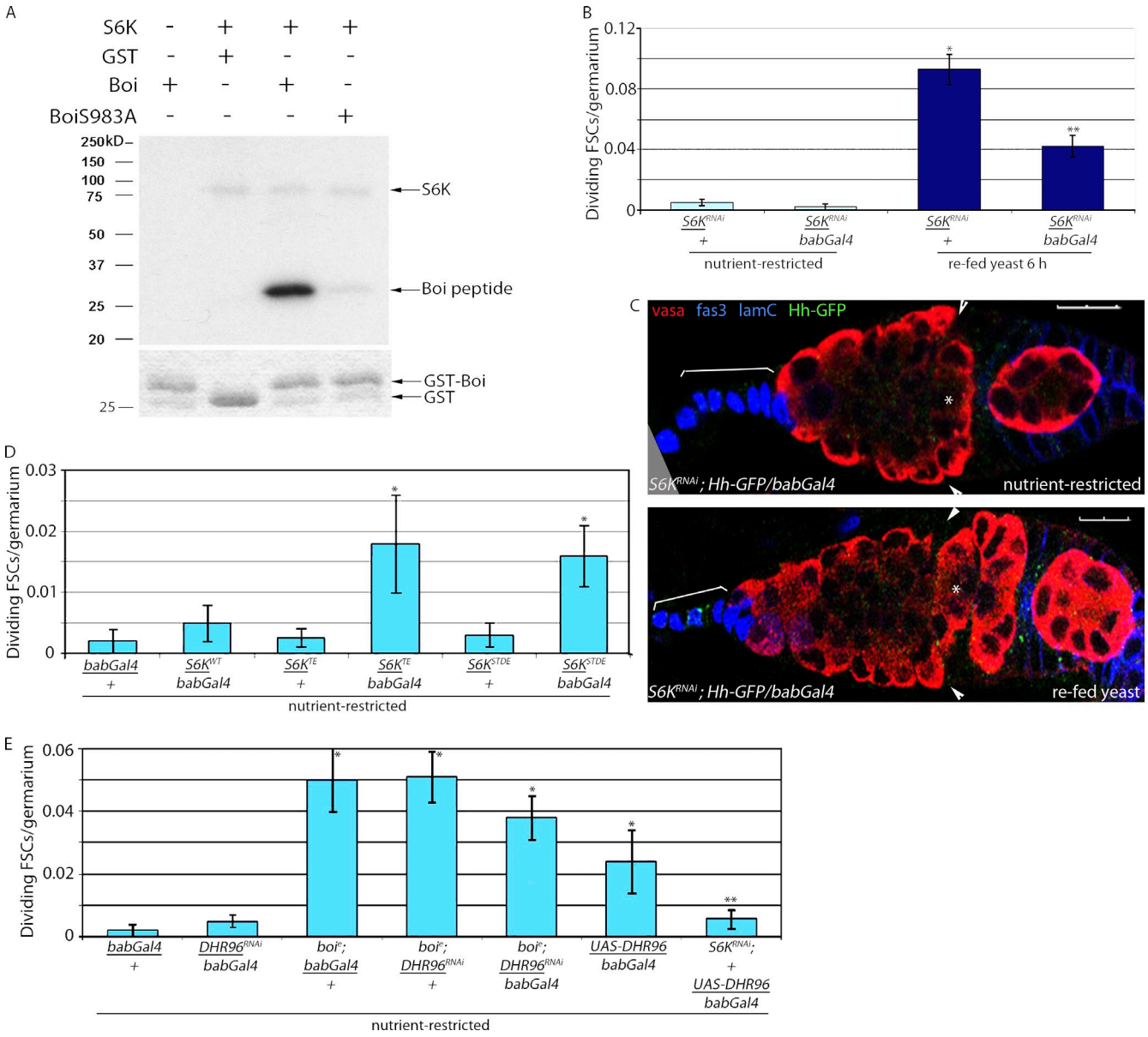

Figure 8. **Stimulation of FSC proliferation after refeeding is S6K dependent.** (A) In the presence of human S6K, Boi^{S983} is phosphorylated (top, third lane, bottom band). Mutation of S983 to A abrogates phosphorylation (top, forth lane), indicating that S983 is the primary site of phosphorylation. Autophosphorylation of S6K also is observed (top, second to forth lanes, top band). No signal is observed in the absence of S6K (top, first lane) or when GST alone is used as a substrate (top, second lane). (bottom) Coomassie-stained gel showing levels of GST (second lane) or GST-Boi (first, third, and forth lanes) used in the assay. (B) Nutrient-restricted *S6K^{RNAi}/bab-Gal4* and *S6K^{RNAi}/+* flies were refed yeast for 6 h. Mean numbers of dividing FSCs (PH3+) per germarium are shown. *, P < 0.00001 versus nutrient-restricted *S6K^{RNAi}/+*. **, P < 0.00001 versus refed *S6K^{RNAi}* (n = 493–1,037; Table 1). (C) *S6K^{RNAi}* flies expressing HhGFP in apical cells (*S6K^{RNAi}; HhGFP/bab-Gal4*) fail to accumulate Hh-GFP in FSCs after refeeding. Hh-GFP, follicle cells (Fas3), apical cells (Lamin C [lamC]), and germ cells (Vasa) are shown. Asterisks indicate flattened germline cyst at the region 2A/2B border. Arrowheads indicate FSCs. Brackets indicate apical cells. Bars, 10 µM. (D) Activated S6K (*S6K^{TE}/bab-Gal4*, *S6K^{STDE}/bab-Gal4*, *S6K^{WT}/bab-Gal4*, and controls *S6K^{TE}/+ and S6K^{STDE}/+*) flies were nutrient restricted for 3 d. Mean numbers of dividing FSCs (PH3+) per germarium are shown. *, P < 0.02 versus nutrient-restricted *bab-Gal4/+* (n = 277–1,132; Table 1). (E) *boi^{e}; DHR96^{RNAi}/bab-Gal4*, *S6K^{RNAi}/+; UAS-DHR96/bab-Gal4*, and control flies were nutrient restricted for 3 d. Mean numbers of dividing FSCs (PH3+) per germarium are shown. *, P < 0.005 versus nutrient-restricted *bab-Gal4/+*. **, P < 0.03 versus nutrient-restricted *UAS-DHR/bab-Gal4* (n = 251–1,014; Table 1). Error bars represent SEs.

suppressed FSC proliferation and Hh release upon refeeding (Fig. 8, B and C; and Table 1). Conversely, expression of forms of S6K bearing mutations that promote the open active, conformation of the kinase (S6K^{TE} and S6K^{STDE}) were sufficient to drive Hh-GFP release and FSC proliferation modestly in nutrient-restricted flies (Fig. 8 D and Table 1). S6K activity is regulated by dietary lipids (Castañeda et al., 2012) and by DHR96 in fly cells (Horner et al., 2009; Lindquist et al., 2011). Moreover, vertebrate orthologues of DHR96, including the vitamin D receptor, regulate S6K activity (Bettoun et al., 2002, 2004). Collectively, these data suggest that feeding stimulates DHR96-dependent activation of S6K and triggers Hh release through phosphorylation of Boi^{S983}. Genetic epistasis experiments support this model. First, the FSC proliferation observed in starved flies overexpressing

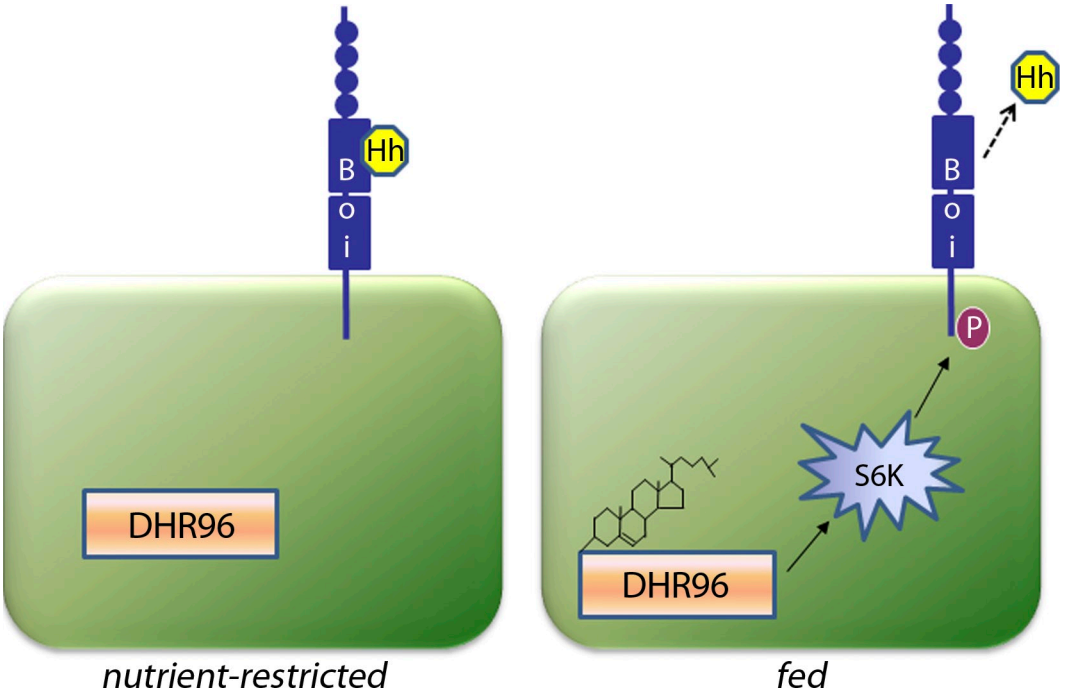

Figure 9. **Cholesterol activation of DHR96 leads to S6K-dependent phosphorylation of Boi^{S983A}, causing release of Hh from apical cells and activation of FSC proliferation.** (left) Hh is sequestered by Boi in nutrient-restricted flies. Upon feeding, cholesterol binds to DHR96 and promotes phosphorylation (P) of Boi^{S983} via S6K activation.

DHR96 in apical cells is suppressed by reduced expression of S6K (*S6K^{RNAi}/+;UAS-DHR96/bab-Gal4*; Fig. 8 E). Second, FSCs proliferate in nutrient-restricted *boi* mutants bearing reduced expression of DHR96 or S6K, indicating that Hh release from Boi is sufficient to drive FSC proliferation in the absence of critical upstream regulators (Fig. 8 E and Table 1).

Discussion

Clear benefits of dietary restriction have been demonstrated for age-related decline in stem cell function and cancer initiation and progression, implicating nutrient signals in their progression (Longo and Fontana, 2010; Omodei and Fontana, 2011). Recent work has uncovered molecular pathways that contribute to the benefits of a healthy diet (Fontana et al., 2010), but little is known about the mechanisms that interpret specific nutritional signals to control stem cell behavior. Here, we have defined a multistep molecular pathway that interprets nutritional signals to control epithelial stem cell proliferation in the fly ovary (Fig. 9). In the absence of nutrients, Boi sequesters Hh on the surface of apical cells, preventing Hh-mediated stimulation of FSC proliferation in conditions that are unfavorable for egg production. Upon feeding, increased dietary cholesterol levels are sensed by apical cells via DHR96. DHR96 then activates S6K, triggering phosphorylation of Boi^{S983} and reducing the ability of Boi to sequester Hh on the surface of apical cells, leading to Hh release. After release, Hh is delivered to FSCs, where it stimulates FSC proliferation in a Smo-dependent manner (Fig. 9). Potential conservation of this signal relay model in mammalian tissues will have clear implications for developing cancer therapies via inhibition of growth factor release, improving regenerative medicine strategies, and understanding normal processes, such as aging, that depend on maintenance of healthy adult stem cell populations.

Our data indicate that Boi must serve two important functions to control FSC proliferation. First, it must sequester Hh molecules, preventing them from reaching FSCs when conditions are unfavorable for egg production. Second, Boi must release Hh molecules when abundant food is present, to drive FSC proliferation rapidly and efficiently. All forms of Boi that are capable of binding to Hh rescue the ability of Boi to sequester Hh on apical cells, supporting previous observations that Hh binding is necessary for Boi function (Fig. 7 A; Yao et al., 2006; McLellan et al., 2008; Beachy et al., 2010; Hartman et al., 2010; Zheng et al., 2010). In contrast, our results indicate that the Boi cytoplasmic domain is critical for Hh release (Fig. 7 B). The ability of Boi to promote feeding-dependent FSC proliferation is dramatically weakened upon cytoplasmic domain deletion or mutation of the S6K target site. These results support the model that a feeding-dependent, inside-out signaling mechanism reduces the ability of Boi to sequester Hh. By analogy to the well-studied effects of inside-out signaling on integrin conformation (Margadant et al., 2011; Ye et al., 2011), the simplest model to explain this requirement is that S983 phosphorylation alters Boi conformation, weakening Boi-Hh affinity and promoting Hh release.

Several observations suggest that Hh release is a primary mechanism for stimulating stem cell proliferation in response to dietary changes. First, loss of Smo activity within FSCs dramatically suppresses proliferation stimulated by yeast or cholesterol (Fig. 4, A and B), demonstrating that Hh signaling is required within stem cells for feeding-stimulated proliferation.

Second, Hh accumulates rapidly within FSCs upon feeding, in a dynamic localization pattern that correlates precisely with feeding-stimulated FSC proliferation (Fig. 2). Finally, the remarkable ability of FSCs and GSCs in *boi* mutants to divide in the absence of dietary protein, lipid, complex carbohydrates, vitamins, or minerals (Fig. 5) strongly supports a model in which Hh release drives ovarian stem cell proliferation regardless of the nutritional status of the organism. The response may be extremely rapid as a result of the efficient absorption of dietary cholesterol (Horner et al., 2009) coupled with the presence of Hh poised for release on the surface of apical cells (Figs. 2, 3, and S3; Hartman et al., 2010).

One appealing possibility is that this mechanism coordinates GSC and FSC divisions after a period of starvation. According to this model, cholesterol targets a single cellular source (apical cells) to promote Hh release and stimulate proliferation of both stem cell populations simultaneously rather than requiring a complex interpretation of one or more dietary signals by each stem cell individually. This rapid, coordinated mechanism may promote initial follicle production until the slower process of protein and complex carbohydrate digestion elevates systemic insulin levels to maintain steady-state rates of egg production (Drummond-Barbosa and Spradling, 2001; LaFever and Drummond-Barbosa, 2005, O'Brien et al., 2011). This model is supported by our observations that reduced expression of InR in apical cells suppressed proliferation in well-fed, steady-state flies but had no effect on FSC or GSC proliferation upon feeding of nutrient-restricted flies (Fig. S5).

The role of Hh likely differs in the presence of abundant food. Under normal feeding conditions, Hh signaling is not required for GSC proliferation but is still essential for FSC proliferation control (Forbes et al., 1996a; King et al., 2001; Zhang and Kalderon, 2001). Moreover, *boi* mutants exhibit excess FSC proliferation even when raised on a normal diet (Hartman et al., 2010). Together, these observations suggest Boi may act as a rheostat under steady-state conditions, translating systemic dietary cholesterol levels to modulate Hh release and FSC proliferation. Defining how Hh is delivered to the FSC niche and processed by FSCs to promote their proliferation will provide insight into the contribution of this mechanism under both refed and steady-state conditions.

Recent work from several laboratories has shown that changes in nutritional status can have dramatic effects on stem cell proliferation, maintenance, and self-renewal. In the cases reported so far, diet-dependent changes in insulin signaling affect stem cells directly (LaFever and Drummond-Barbosa, 2005; Mairet-Coello et al., 2009; Chell and Brand, 2010; McLeod et al., 2010; Michaelson et al., 2010; Sousa-Nunes et al., 2010; O'Brien et al., 2011) or by altering signaling events within components of the stem cell niche or differentiating daughter cells (Hsu and Drummond-Barbosa, 2009a; Mathur et al., 2010; McLeod et al., 2010). Insulin release from producing cells in mammals has mechanistic similarities to nutrient-stimulated Hh release in the fly ovary because insulin is sequestered in vesicles at the surface of producing cells and released when increased local glucose levels promote rapid and efficient secretion (Rutter and Hill, 2006). However, our results demonstrate that the primary nutrient required for feeding-stimulated FSC proliferation is dietary cholesterol. In flies, absorption of dietary cholesterol occurs in the midgut, the equivalent of the mammalian small intestine (Voght et al., 2007). The nuclear hormone receptor DHR96 binds directly to cholesterol (Horner et al., 2009) and maintains cholesterol and triacylglycerol homeostasis through transcriptional regulation of genes involved in lipid metabolism in absorptive cells of the larval midgut (Horner et al., 2009; Sieber and Thummel, 2009, 2012; Bujold et al., 2010). Our results are consistent with a model in which DHR96 also functions within apical cells of the ovary as a sensor of changes in systemic cholesterol levels (Fig. 3). DHR96-mediated cholesterol homeostasis might control membrane composition within apical cells or expression of transcriptional targets that promote S6K activation, resulting in Hh release through phosphorylation of the Boi C terminus.

Initially, it was also possible that some of the effects of diet on Hh-stimulated FSC proliferation might be caused by altered cholesterol modification of the Hh protein. Full-length Hh precursor proteins are cleaved during transit through the Golgi followed by the addition of cholesterol to the newly generated C-terminal ends of active Hh ligand, a mechanism that is known to control Hh diffusion across tissues and liposome-dependent delivery to receiving cells (Porter et al., 1996; Guerrero and Chiang, 2007; Eaton, 2008). However, a form of Hh that cannot be modified by cholesterol (Hh-N–GFP) was sequestered in starved flies and exhibited a time course of release and accumulation in FSCs upon feeding that was nearly identical to that observed for cholesterol-modified Hh (Hh-GFP; Fig. 4). The primary difference between unmodified and cholesterol-modified Hh was in the timing of Hh reaccumulation on the surface of apical cells after a feeding-stimulated release (Fig. 4). Although the role of cholesterol modification in Hh delivery to FSCs has not yet been addressed, these results suggest that the primary role of dietary cholesterol in apical cells is to trigger release of mature, cholesterol-modified Hh molecules sequestered outside of apical cells rather than to modify nascently generated Hh ligand on the inside.

In addition to fly ovarian stem cells, epithelial stem cells in the fly midgut and neuroblasts in developing fly embryos proliferate in response to changes in the nutritional status of the organism via a multistep pathway (Chell and Brand, 2010; Sousa-Nunes et al., 2010; O'Brien et al., 2011). In both cases, systemic signals induce locally produced growth factors to stimulate stem cell proliferation in a paracrine fashion. Proliferation of mammalian neural stem cells also is sensitive to changes in nutritional status (Spéder et al., 2011). These cells proliferate in response to stimulation with Sonic Hh (Traiffort et al., 2010), suggesting the possibility that feeding-triggered Sonic Hh release might be conserved in this tissue. Progenitor cell populations in other nutrient-responsive tissues such as the liver also proliferate in response to Sonic Hh signaling (Sánchez and Fabregat, 2010), but the connection between dietary changes and Hh signaling have not been examined. Finally, this mechanism may contribute to human conditions such as cholesterol metabolism disorders, aging-related decline in tissue function, or cancer initiation and progression, in which Hh signaling and

diet may be linked (Longo and Fontana, 2010; Omodei and Fontana, 2011; Porter and Herman, 2011). If conserved in mammalian tissues, our results suggest that some of the benefits of a low calorie diet occur because of reduced levels of growth factor release, resulting in reduced proliferation and extended life span of normal stem cell populations.

Materials and methods

Fly strains

boi^{e01708} (*boi*^{e}) was generated by Exelixis and is maintained by the Harvard stock center. *boi*^{e} is a loss-of-function allele expressing 0.2% of WT, full-length *boi* transcript in the ovary (Hartman et al., 2010). Ihog was expressed in apical cells by generating female flies of the genotype *ihog*^{EP} (*P{EP}ihog*^{G13202})/+; *bab-Gal4/+*. UAS-S6K^{TE} and UAS-S6K^{STDE} (Barcelo and Stewart, 2002) bear mutations in phosphorylation sites known to be important for maintaining an open, active conformation. Specifically, mutation of T398 to glutamic acid (S6K^{TE}) mimics phosphorylation of this residue by target of rapamycin kinase opening the linker domain of S6K for subsequent activation. Similarly, mutation of two phosphorylation sites in the autoinhibition domain of S6K, S418 to aspartic acid and T422 to glutamic acid (S6K^{STDE}), stabilizes the open conformation and enables kinase activation via additional phosphorylation events (Barcelo and Stewart, 2002). RNAi directed against *smo* (*P{UAS-smoRNAi}2P{UAS-smoRNAi}*), *hh* (*P{TRiP.JF01804}attP2*), *DHR96* (*P{TRiP.JF02350}attP2*), *S6K* (*P{KK107986}VIE-260B*), or *InR* (*P{TRiP.JF01183}attP2* or *P{TRiP.JF01482}attP2*) was expressed either in apical cells using *bab-Gal4* (*P{GawB}bab1{Pgal4-2}*) or *109-53-Gal4* (*P{GawB} 109-53*) or in FSCs and their progeny using *109-30-Gal4* (*P{GawB}109-30*). UAS-DHR96 (*EP-DHR96(P{EPgy2}Hr96*^{EY0217}) was overexpressed in apical cells using *bab-Gal4*. Clonally marked FSCs were generated using the MARCM (Mosaic Analysis with a Repressible Cell Marker) system (Lee and Luo, 2001) with 109-30-Gal4 (*Gal80 19AFRT Flp122/19AFRT; 109-30-Gal4/UAS-GFP*).

Transgenic fly lines

pUASt-boi (*UAS-boi*) was generated by cloning the full-length *boi* transcript *boi-RB* from pOT2-SD07678 (available at GenBank under accession no. AY061833; Drosophila Genomics Resource Center) into *pUASt*. *pUASt-boiΔFN1* was created by site-directed excision of bases corresponding to amino acids 456–598 of Boi (Hartman et al., 2010). In *pUASt-boi*$^{\Delta cyto}$, amino acids 754–997 of Boi were deleted, and in *pUASt-boi*$^{\Delta c\text{-}term}$, amino acids 971–997 of Boi were deleted. In *pUASt-boi*^{S983A}, serine 983 was mutated to alanine. Amino acid numbers are from Boi isoform B (available at RefSeq under accession no. NP_726811). *pUASt-Hh-GFP* (*Hh-GFP*) was generated by cloning full-length PCR-amplified Hh into pDONR. *pml1*-digested EGFP PCR amplified from pTWG (Drosophila Genomics Resource Center) was inserted into *pml1*-digested pDONR-Hh. The *pml1* restriction site falls directly before the site where full-length Hh is cleaved and modified by the addition of cholesterol, resulting in a GFP-tagged, cholesterol-modified active Hh molecule (Torroja et al., 2004). *pUASt-Hh-N–GFP* (*Hh-N–GFP*) was generated by cloning PCR-amplified Hh-N (amino acids 1–257) into pDONR. Hh-N is an active form of Hh that lacks cholesterol modification. Hh-GFP and Hh-N–GFP were then transferred into pTW and pTWG. All transgenic fly lines were created using the *Drosophila* cloning system (Gateway; Carnegie Institution of Washington). Transgenic flies were generated by BestGene, Inc.

Nutritional assays

Nutrient restriction was performed as follows: Flies were raised on fruit juice plates containing only simple sugars (50% grape juice, 3% bacto agar, 1% glacial acetic acid, and 1% methyl paraben) and stimulated by feeding with yeast or yeast extract supplemented with 0.2 mg/g cholesterol in EtOH or EtOH only for the indicated times. Egg numbers were counted every 24 h in triplicate. TUNEL assay was performed using in situ apoptosis detection kit (ApopTag red; EMD Millipore).

Antibody generation

Polyclonal anti–FC-NA antibodies were developed from the injection of an antigen consisting of full-length GST fused to three amino acids, A-E-R, into Sprague-Dawley rats. The resulting antiserum marks an unidentified antigen expressed at high levels in all follicle cells, including FSCs, and at much lower levels in other cells within the ovary.

Immunofluorescence

Fly ovaries were prepared as previously described (Hartman et al., 2010). In brief, flies were dissected in Grace's insect medium (Sigma-Aldrich), fixed in 4% paraformaldehyde for 10 min at room temperature, washed three times for 10 min in PBS-T (PBS with 0.3% Triton X-100), and incubated with primary antibody in PBS-T with 0.5% BSA for 2 h at room temperature. Ovaries were washed three times for 10 min in PBS-T and incubated with secondary antibody in PBS-T with 0.5% BSA for 2 h at room temperature. Ovaries to be stained with Boi antibody were fixed in 2% formaldehyde on ice for 10 min. WT and mutant ovaries were compared directly by dissecting, fixing, and immunostaining with premixed primary and secondary antibodies at the same time. Primary antibodies were rat anti-Boi (1:50), rabbit anti-Vasa (1:2,000; Hay et al., 1990); rabbit anti-Hh (a gift from P. Therond , Institut Valrose Biologie, Nice, France; 1:100; Gallet et al., 2003), goat anti-Hh (1:100; Santa Cruz Biotechnology, Inc.), mouse anti-Fas3 (1:25; Developmental Studies Hybridoma Bank; Patel et al., 1987), mouse anti–Lamin C (1:100; Developmental Studies Hybridoma Bank; Riemer et al., 1995), rat anti–FC-NA (1:2,000), chicken anti-GFP (1:1,000; Invitrogen), or rabbit antiphospho–histone-H3 (1:1,000; EMD Millipore). Secondary antibodies used were FITC, Cy3, and Cy5 conjugated to species-specific secondary antibodies (Jackson ImmunoResearch Laboratories, Inc.). Samples were mounted in Vectashield mounting medium (Vector Laboratories). Images were collected at room temperature (approximately 22°C) using 40×, 1.25 NA or 63×, 1.4 NA oil immersion lenses (Leica) on an upright microscope (DM5000 B; Leica) coupled to a confocal laser scanner (TCS SP5; Leica). LAS AF SP5 software (Leica) was used for data acquisition. Images representing individual channels of single confocal slices from each germarium were exported as TIFF files, and images were converted to figures using Photoshop software (Adobe).

p70S6K assay

0.2 µg active p70S6K (R&D Systems) was incubated with 800 µM ATP/γ-[^{32}P]ATP in kinase buffer and 3 µg GST, GST-Boi, or GST-Boi^{983A} peptides (amino acids 973–998) at 30°C for 30 min, and reactions were analyzed by SDS-PAGE/Coomassie blue staining and autoradiography.

Statistics

Dividing FSCs were determined by scoring 215–2,100 germaria for phospho–histone-H3–positive FSCs per germarium. FSCs were identified by their location at the border of germarial regions 2A and 2B, low level expression of Fas3 (Fas3^{lo}), a marker for prefollicle cells, and the presence of a triangular nucleus, a feature that distinguishes FSCs from their daughter cells and neighboring escort cells ((Nystul and Spradling, 2007). Student's *t* tests for two samples were used, with significance achieved at $P \leq 0.05$. The Bonferroni method was used for feeding experiments in which multiple tests were run on associated data.

Online supplemental material

Fig. S1 shows WT flies survive up to 75 d on nutrient-restricted diets. Fig. S2 shows expression patterns of Gal4 drivers in germaria. Fig. S3 shows localization of endogenous Hh in nutrient-deprived and refed WT flies. Fig. S4 shows FSCs can be identified by specific characteristics. Fig. S5 shows loss of InR in apical cells does not block FSC proliferation after refeeding. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201212094/DC1. Additional data are available in the JCB DataViewer at http://dx.doi.org/10.1083/jcb.201212094.dv.

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