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Polished Rice Regulates Maturation but Not Survival of Secondary Cells in *Drosophila* Male Accessory Gland

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ABSTRACT

In *Drosophila* males, the accessory gland is responsive to nutrient signal-dependent regulation of fertility/fecundity. The accessory gland is composed of two types of binucleated epithelial cells, about 1000 main cells and 60 secondary cells (SCs). The transcription factors Defective proventriculus (Dve), Abdominal-B, and Ecdysone receptors (EcRs) are strongly expressed in adult SCs. In response to nutrient conditions during development, coordinated action between Dve and ecdysone signaling determines the optimal number of SCs and regulates their maturation. A downstream effector of ecdysone signaling, Ftz-F1, is crucial in this process. Another downstream effector, Polished rice (Pri), is small peptides of 11 or 32 amino acids. Here we show that *pri* is required for maturation of SCs and for male fecundity, whereas it is not involved in determination of the number of SCs. We provide evidence that Pri acts downstream of Ftz-F1 to regulate maturation but not survival of SCs.

1 | Introduction

Steroid hormones play various physiological roles, including metabolism and reproduction in mammals (Deroo and Korach 2006; Mani et al. 2012). Steroid hormones in insects are ecdysteroids, and the major form in *Drosophila melanogaster* is ecdysone. Ecdysone signaling activates specific target genes such as the orphan nuclear receptor Ftz-F1 to regulate the precise timing of development, including larval molting and metamorphosis (Faunes and Larrain 2016; Huang et al. 2008; Moeller et al. 2017; Petryk et al. 2003; Rewitz et al. 2013). Ecdysone signaling also activates a target gene, *polished rice* (*pri*), which encodes small peptides of 11 or 32 amino acids (Kondo et al. 2007), to regulate epidermal and tracheal morphogenesis during embryonic development (Kondo et al. 2010; Pi et al. 2011; Taira et al. 2021).

Small open reading frames (smORFs) have recently been recognized as a new class of essential genes and are also evolutionarily conserved (Khitun et al. 2019; Plaza et al. 2017; Pueyo et al. 2016; Saghatelian and Couso 2015). Pri small peptides act as temporal mediators of ecdysone signaling, and the *pri* gene is also known as *tarsal-less* and *mille-pattes*, which is involved in leg morphogenesis and embryonic patterning (Albuquerque et al. 2015; Galindo et al. 2007; Gallois et al. 2024; Ray et al. 2019).

In adult males, ecdysone is thought to be synthesized in the accessory gland, and the ecdysone signaling is required for male fertility (Hentze et al. 2013; Matsuka et al. 2024; Sharma et al. 2017). The accessory gland is composed of two types of binucleated epithelial cells, about 1000 main cells and 60 secondary cells (SCs) in each lobe. Adult main cells are flat hexagonal cells, and SCs are large spherical cells clustered at the distal tip

of each accessory gland lobe. These cells secrete Accessory gland proteins (Acps) as seminal fluid components by which trigger the female post-mating responses to enhance male fertility/fecundity. Binucleation is achieved by inhibition of cytokinesis and contributes to plastic changes of cell shape for effective ejection of seminal fluid (Matsuka and Nakagoshi 2019; Taniguchi et al. 2012, 2018).

The homeodomain transcription factor Defective proventriculus (Dve) is involved in various physiological functions such as morphogenesis, cell-type specification, and functional differentiation (Johnston Jr. et al. 2011; Kitamata et al. 2024; Nakagawa et al. 2011; Nakagoshi et al. 2002; Shirai et al. 2007; Yorimitsu et al. 2011). Dve is highly expressed in SCs in the accessory gland, and loss of Dve activity induces cell death of SCs and results in decreased fecundity (Minami et al. 2012). SCs have unusually large vacuoles, which are nonacidic secretory vacuoles (SVs) and acidic mature late endosomal multivesicular bodies or lysosomes (MVBLs) (Redhai et al. 2016). Exosomes, nano-sized vesicles generated in the SVs, are secreted into the seminal fluid and induce a part of female postmating responses (Corrigan et al. 2014). SCs are a minor population in the accessory gland, whereas their survival and maturation are essential for male fecundity.

Nutrient signaling during pupal development induces Dve expression, and its level over a threshold in a restricted period in the mid-pupal stage (critical period) is maintained. This stable expression becomes independent of nutrient signaling and protects immature SCs from cell death (Kubo et al. 2018; Matsuka et al. 2024). This process determines the optimal number of SCs in response to nutrient conditions during the critical period, and it also requires EcR signaling together with a downstream effector, Ftz-F1, through a positive feedback loop. After the determination of the optimal number of SCs, in the late pupal stage, they increase cell volume with the accumulation of large vacuoles. This maturation process is regulated by nutrient signal-dependent EcR signaling and the activity of the downstream transcription factor Abdominal-B (Abd-B) (Gligorov et al. 2013; Leiblich et al. 2019; Matsuka et al. 2024). Here, we provide evidence that Pri small peptides act differently for the survival and maturation of SCs.

2 | Results

2.1 | Polished Rice Is Required for Maturation of Secondary Cells

Secondary cells (SCs) in the *Drosophila* accessory gland are important for regulating male fertility (Gligorov et al. 2013; Minami et al. 2012) and the number of SCs is optimized by the transcriptional regulator Dve in response to nutrient conditions (Kubo et al. 2018). Determination of the number and subsequent maturation of SCs requires ecdysone signaling, which is mediated by the downstream effector Ftz-F1 (Matsuka et al. 2024). It remains unclear whether another downstream effector Polished rice (Pri) is also involved in SC development. Thus, we tested the effects of *pri* knockdown (KD) on accessory gland development.

pri KD from the mid-pupal stage (*dG30A>pri-IR*) showed a size reduction of SCs (Figures 1A,B,E and 2A,C). The expression of Abdominal-B (Abd-B), which is involved in SC maturation, was also reduced (Figures 1C,D and 2B,D, Figure S1A). However, the number of SCs was unaffected (Figures 1F and 2J). Similar results were obtained with another GAL4 driver line, *prd-GAL4* (Figure 1E,F, *prd>pri-IR*), and these phenotypes were the same as for the *ftz-F1* KD (Matsuka et al. 2024).

Cell-fate determination of accessory gland primordia depends on fibroblast growth factor (FGF) signaling, and the mesodermal cells expressing an FGF receptor, Breathless (Btl), are recruited into a part of the male genital disc during late larval development (Ahmad and Baker 2002). The *btl*-expressing cells become epithelial and give rise to accessory glands and seminal vesicles. Thus, expression of *breathless (btl)-GAL4* starts earlier than those of *prd-GAL4* and *dve-GAL4*. When ecdysone signaling is inhibited from the early stage of accessory gland development by using *btl-GAL4* (*btl>EcR[DN], btl>ftz-F1-IR*), a marked reduction in the number of SCs is observed (Matsuka et al. 2024). The number of SCs is regulated by Dve and EcR signaling through apoptotic cell death (Kubo et al. 2018; Matsuka et al. 2024) (Figure S2). Therefore, we examined the possibility that the Pri activity is also required to determine the number of SCs. Unexpectedly, *pri* KD (*btl>pri-IR*) did not affect the number of SCs and cell size was also unaffected (Figure S3). Additionally, the expression of Abd-B was the same level between control and *pri* KD (Figure S1B,B′,D,D′). It is assumed that these phenotypes are due to low level expression of *btl-GAL4* after the mid-pupal stage compared to those of *dve-GAL4* and *prd-GAL4*. Taken together, these results strongly suggest that the number of SCs is regulated by Dve and EcR signaling independent of the Pri activity, and that the size of SCs is regulated by EcR signaling that requires the Pri activity.

Ecdysone signaling regulates not only SC maturation but also main cell development, because the fertility of *EcR* KD males is greatly reduced compared to that of *dve* KD males, which have no SCs (Matsuka et al. 2024). Therefore, we assessed the fertility of *pri* KD males. As a result, the number of progenies produced by females that mated with *pri* KD males was clearly reduced (Figure S4). However, it was significantly higher compared to females that mated with *dve* KD males, suggesting that the Pri activity is mainly required for SC maturation.

2.2 | Forced Expression of *Pri* Rescues the Lack of EcR Signaling

The epidermal phenotype of the ecdysone-deficient mutant can be rescued by forced expression of *pri* (Chanut-Delalande et al. 2014; Taira et al. 2021). In addition, SC size reduction associated with reduced ecdysone signaling can be rescued by forced expression of *ftz-F1* (Matsuka et al. 2024). Since *ftz-F1* KD and *pri* KD show the same phenotype in regulating SC size (maturation), it seems likely that SC size reduction upon inhibition of ecdysone signaling is also rescued by *pri* expression. Forced expression of *pri* under the EcR-inhibited conditions resulted in marked recovery of SC size (Figures 1G and S5A–D). However, other phenotypes such as mononucleation and reduced number of SCs were not recovered (Figures 1H and S5A–D). In contrast, forced expression of *ftz-F1* under

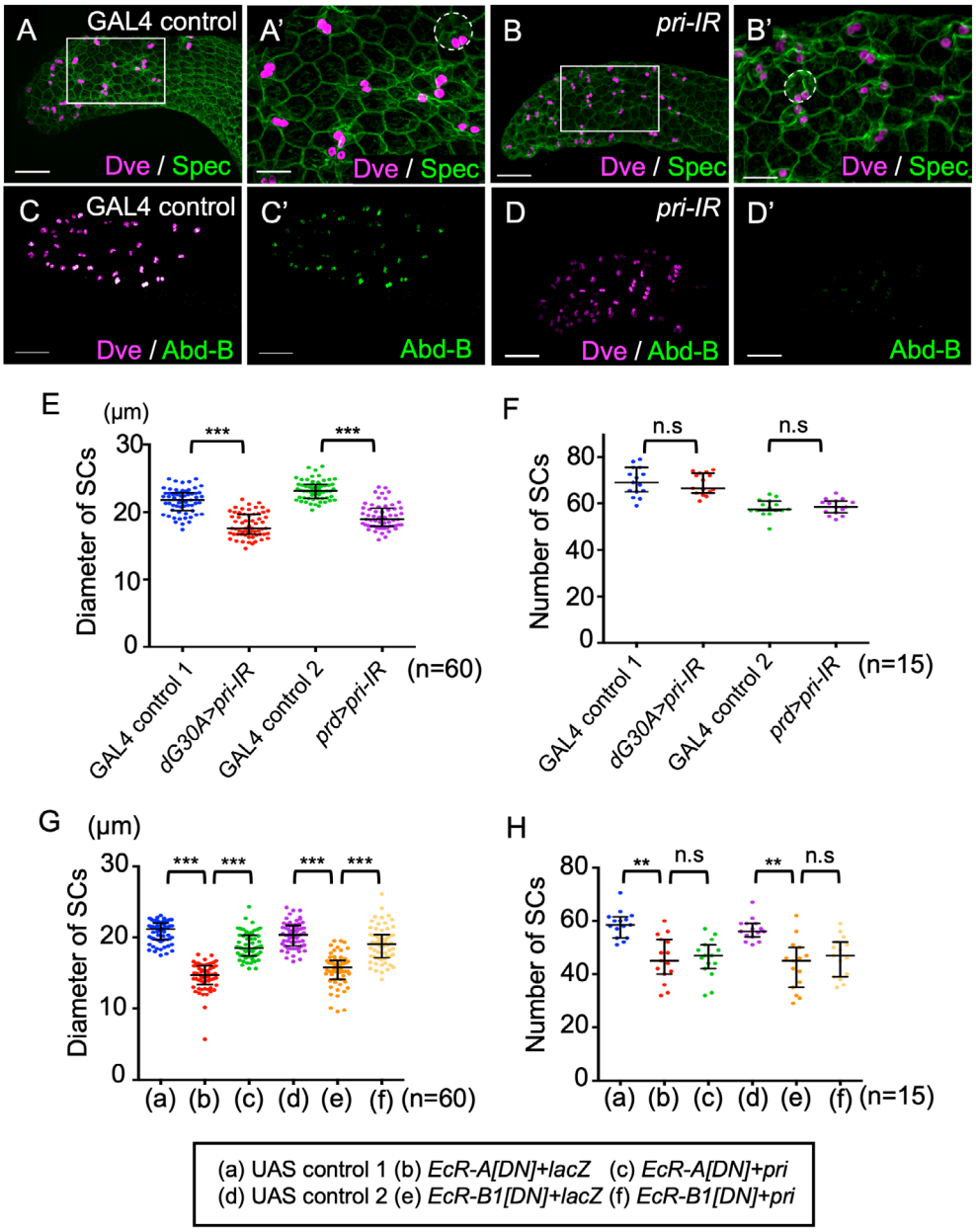

FIGURE 1 | Polished rice is required for maturation of secondary cells. (A–D) Inhibition of *pri* impairs secondary cell (SC) development. Accessory glands of adult day5 males of *dve-GAL4[30A]/+* (A, C; GAL4 control 1) and *dve-GAL4[30A]> UAS-pri-IR* (B, D; *dG30A>pri-IR*). Expression patterns of Spectrin and Abd-B (green), and Dve (magenta) are shown. Magnified views of the boxed region (A and B) are shown in (A′ and B′), and typical examples of SC are outlined. Single channel images of Abd-B expression are shown in (C′) and (D′). Scale bar: 50 μm (A–D), 20 μm (A′, B′). (E-H) Diameter (E, G) and number (F, H) of SCs in accessory glands of the indicated genotypes: *prd-GAL4/+* (GAL4 control 2), *prd-GAL4> UAS-pri-IR* (*prd>pri-IR*), *UAS-EcR-A[DN]/+; UAS-GFP::LacZ.nls/+* (UAS control 1), *dG30A>UAS-EcR-A[DN] + UAS-GFP::LacZ.nls* (*EcR-A[DN] + lacZ*), *dG30A>UAS-EcR-A[DN] + UAS-pri* (*EcR-A[DN] + pri*), *UAS-EcR-B1[DN]/+; UAS-GFP::LacZ.nls/+* (UAS control 2), *dG30A>UAS-EcR-B1[DN] + UAS-GFP::LacZ.nls* (*EcR-B1[DN] + lacZ*), *dG30A>UAS-EcR-B1[DN] + UAS-pri* (*EcR-B1[DN] + pri*). The number (n) of scored SCs (E, G) and accessory glands (F, H) is indicated, respectively.

the EcR-inhibited conditions partially recovered the number of SCs and their binucleation (Matsuka et al. 2024) (Figure S5E,F). Thus, Ftz-F1 appears to have a higher rescuing activity. However, we cannot exclude the possibility that this difference in rescuing activity simply reflects different levels of UAS sequence-dependent expression. Taken together, results of *pri* KD and rescue experiments indicate that Pri, a downstream effector of ecdysone signaling, is a crucial mediator in the regulation of SC maturation.

2.3 | A Specific Isoform of Ftz-F1 Is Not Necessary for SC Maturation

Ftz-F1 has two isoforms. *α-ftz-F1* is maternally expressed and evenly distributed during early embryonic development, while *β-ftz-F1* is zygotically expressed in the late embryonic and pupal stages (Lavorgna et al. 1993; Ohno and Petkovich 1993). β-Ftz-F1 regulates the timing of larval molting and metamorphosis as a mediator of ecdysone signaling during *Drosophila*

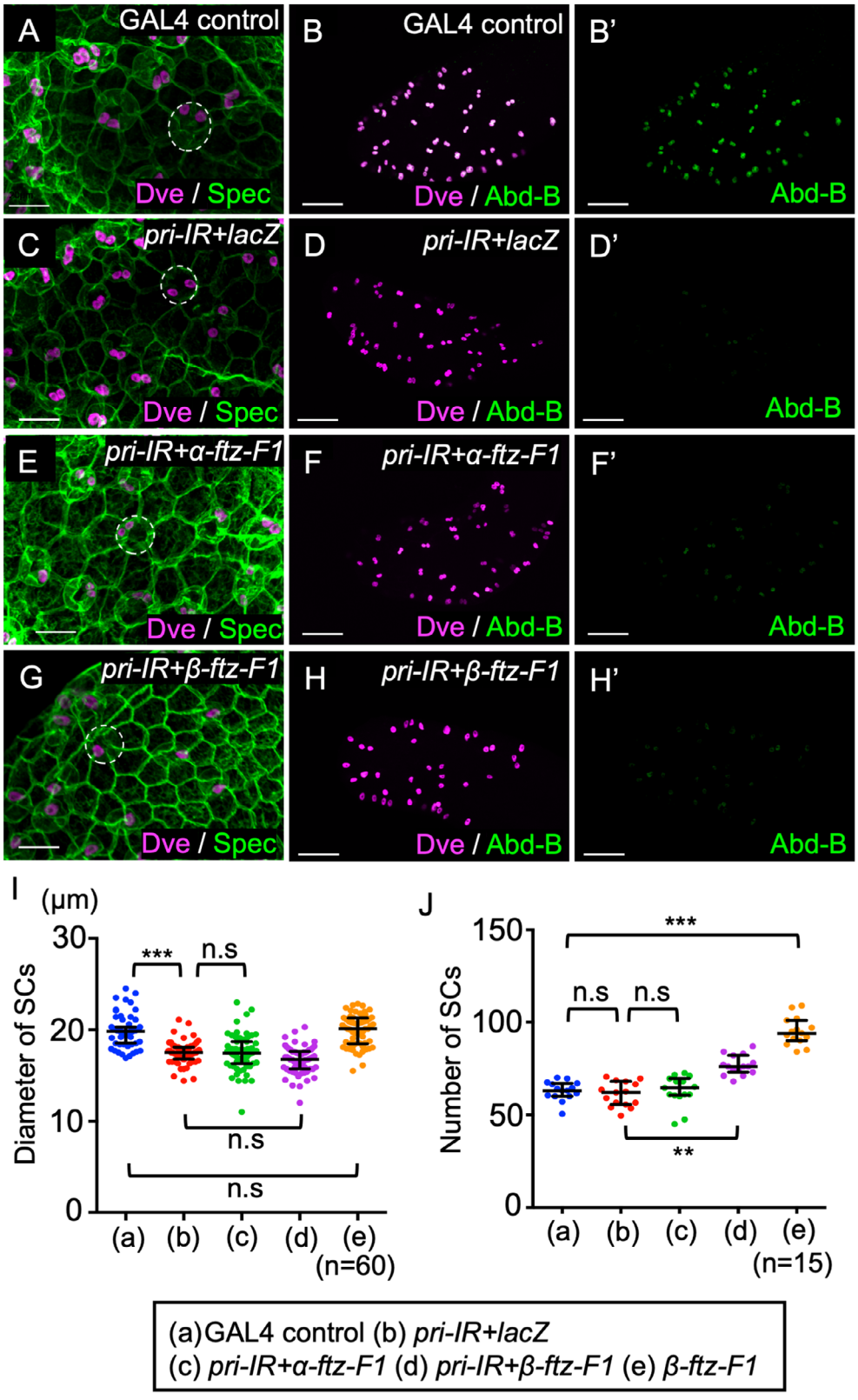

FIGURE 2 | Phenotypes of *pri* KD cannot be rescued with co-expression of *ftz-F1*. (A–H) Accessory glands of adult day5 males of *dG30A/+* (A, B; GAL4 control), *dG30A>UAS-pri-IR + UAS-GFP::LacZ.nls* (C, D; *pri-IR + lacZ*), d*G30A>UAS-pri-IR + UAS-α-ftz-F1* (E, F; *pri-IR + α-ftz-F1*), and *dG30A>UAS-pri-IR + UAS-β-ftz-F1* (G, H; *pri-IR + β-ftz-F1*). Expression patterns of Spectrin and Abd-B (green), and Dve (magenta) are shown. Single channel images of Abd-B expression are shown in (B′, D′, F′, H′). Scale bar: 20 μm (A, C, E, G), 50 μm (B, D, F, H). (I, J) Diameter (I) and number (J) of SCs in accessory glands of the indicated genotypes (a–d). (e) *dG30A>UAS-β-ftz-F1* (*β-ftz-F1*). The number (n) of scored SCs (I) and accessory glands (J) is indicated, respectively.

development (Agawa et al. 2007; Ou and King-Jones 2013). Although the isoform-specific function of Ftz-F1 is unknown, functional overlaps have been reported. For example, segmental abnormalities in early embryos induced by the *α-ftz-F1* KD can be rescued by forced expression of *β-ftz-F1* (Yussa et al. 2001).

Inhibition of ecdysone signaling during the mid-pupal stage leads to size reduction of the entire accessory gland, and this phenotype is rescued by forced expression of *β-ftz-F1*, a major isoform during pupal development (Matsuka et al. 2024). We tested whether this rescuing activity is specific for *β-ftz-F1* or if it can be substituted by *α-ftz-F1*. Forced expression of *α-ftz-F1* under the EcR-inhibited condition (*dG30A>EcR-IR + α-ftz-F1*) recovered the size of SCs with nearly normal size of the accessory gland (Figure S6). Therefore, both of the two isoforms, *α-ftz-F1* and *β-ftz-F1*, have the ability to regulate the size of SCs as downstream effectors of ecdysone signaling.

2.4 | Pri Acts Downstream of Ftz-F1 in SC Maturation

SC size reduction due to inhibition of ecdysone signaling can be rescued by forced expression of *pri* or *ftz-F1* (Figures 1G and S5, S6), suggesting that Pri and Ftz-F1 regulate SC maturation in the same pathway. Thus, we examined epistasis between *ftz-F1* and *pri*. Forced expression of *ftz-F1* under the *pri* KD condition (*dG30A>pri-IR + α-ftz-F1, dG30A>pri-IR + β-ftz-F1*) did not rescue the *pri* KD phenotypes, resulting in reduced size of SCs associated with reduced Abd-B expression (Figures 2 and S1B).

In contrast, forced expression of *pri* under the *ftz-F1* KD condition (*dG30A>ftz-F1-IR + pri*) clearly recovered the size of SCs and their Abd-B expression (Figures 3 and S1C). Thus, the *ftz-F1* KD phenotype was rescued by forced expression of *pri*. If Ftz-F1 and Pri act as independent downstream effectors in parallel pathways, the enhanced Pri pathway might compensate for the lack of Ftz-F1 activity. In this case, cooperative interaction between Ftz-F1 and Pri is expected for maturation of SCs, and it is assumed that the double KD of *ftz-F1* and *pri* results in more severe phenotypes than those of single KD conditions. However, the double KD of *ftz-F1* and *pri* showed no difference in phenotype compared to the single KD of *pri* (Figures 4 and S1D). These results indicate that Pri functions downstream of Ftz-F1.

3 | Discussion

Functional maturation of SCs in the accessory gland is regulated by two steps in response to nutrient conditions. The first step is determination of the optimal number of SCs, and the second step is their maturation. In the first step, nutrient signal-dependent induction of EcR signaling is maintained through the positive feedback loop between EcR and Ftz-F1, and subsequent Dve induction at early pupal stage is changed to stable expression. During the critical period (CP), Dve expression over a threshold level is maintained and becomes to be in a nutrient signal-independent manner. In concert with EcR signaling, these Dve-expressing cells are determined as immature SCs, and this mechanism determines the optimal number of SCs in response to nutrient conditions (Step1 in Figure 5; Matsuka et al. 2024). In the second step, EcR expression in SCs is maintained by the Dve activity, but the expression level still depends on nutrient conditions in the late pupal stage. The level of EcR signaling mediated by Ftz-F1 and Pri regulates appropriate maturation of SCs (Step 2 in Figure 5). This mechanism fine-tunes maturation of SCs in response to nutrient conditions in the late pupal stage.

Our results suggest that the Pri activity is only required for the second step. Because Pri peptides trigger proteasome-mediated protein processing, converting the Shavenbaby (Svb) transcriptional repressor into a shorter activator (Zanet et al. 2015), Pri might change the activity of Ftz-F1 after the critical period. However, forced expression of *pri* under the *ftz-F1* KD condition rescued the size reduction of SCs, suggesting that Pri regulates the maturation of SCs independent of Ftz-F1 activities. As our results suggest that Pri acts downstream of Ftz-F1, another possibility is that the Pri activity is required for the activation of transcription factors for *Abd-B* expression or for the stabilization of Abd-B protein. As Abd-B expression becomes detectable in the accessary gland after the critical period, this is consistent with the result that Pri is not required for the first step.

Both of the two isoforms of Ftz-F1 rescued the size reduction of SCs under the EcR-inhibited conditions. So, it remains unclear whether the major isoform during pupal development, β-Ftz-F1, has specific functions for maturation of SCs. Interestingly, forced expression of *β-ftz-F1*, but not *α-ftz-F1*, increased the number of SCs (Figure 2J). Furthermore, forced expression of *β-ftz-F1* in the wild-type background also showed the increased number of SCs (Figure 2J). The number of SCs is optimized in response to nutrient conditions, which is sensed by levels of nutrient signal-dependent expression of Dve and EcRs during the critical period (Matsuka et al. 2024). Thus, it is assumed that forced expression of *dve* or *β-ftz-F1* reflects a nutrient-rich condition and resulted in the increased number of SCs (Figure 2J). Although the forced expression of *α-ftz-F1*could rescue the EcR-inhibited conditions, it could not increase the number of SCs. These results suggest that β isoform-specific functions of Ftz-F1 are required for maintaining a positive feedback loop between β-Ftz-F1 and EcRs or for the survival activity of immature SCs (Figure 5). The β isoform-specific functions of Ftz-F1 appear to be independent of Pri-mediated ecdysone signaling. Taken together, these results will provide insights into the functional mechanisms of small peptides in the nutrient signal-dependent pathway.

4 | Experimental Procedures

4.1 | *Drosophila* Strains

All flies were reared on a standard yeast and cornmeal-based diet in 12 h light/dark cycles at 25°C. Oregon-R (OR) was used as a wild-type control. The following GAL4/UAS lines were used: *dve-GAL4[30A]* (Minami et al. 2012), *prd-GAL4*, *btl-GAL4, UAS-α-ftz-F1, UAS-β-ftz-F1, UAS-EcR-A[DN](W650A), UAS-EcR-B1[DN](W650A)* (Bloomington Drosophila Stock Center), *UAS-EcR-IR* (Vienna Drosophila Resource Center, v37058), *UAS-GFP::lacZ.nls* (Kyoto Drosophila Stock Center, DGRC #109130, #109131), *UAS-ftz-F1-IR* (Sultan et al. 2014), *UAS-β-ftz-F1* (Akagi et al. 2016), *UAS-pri-IR*, and *UAS-pri* (Kondo et al. 2007).

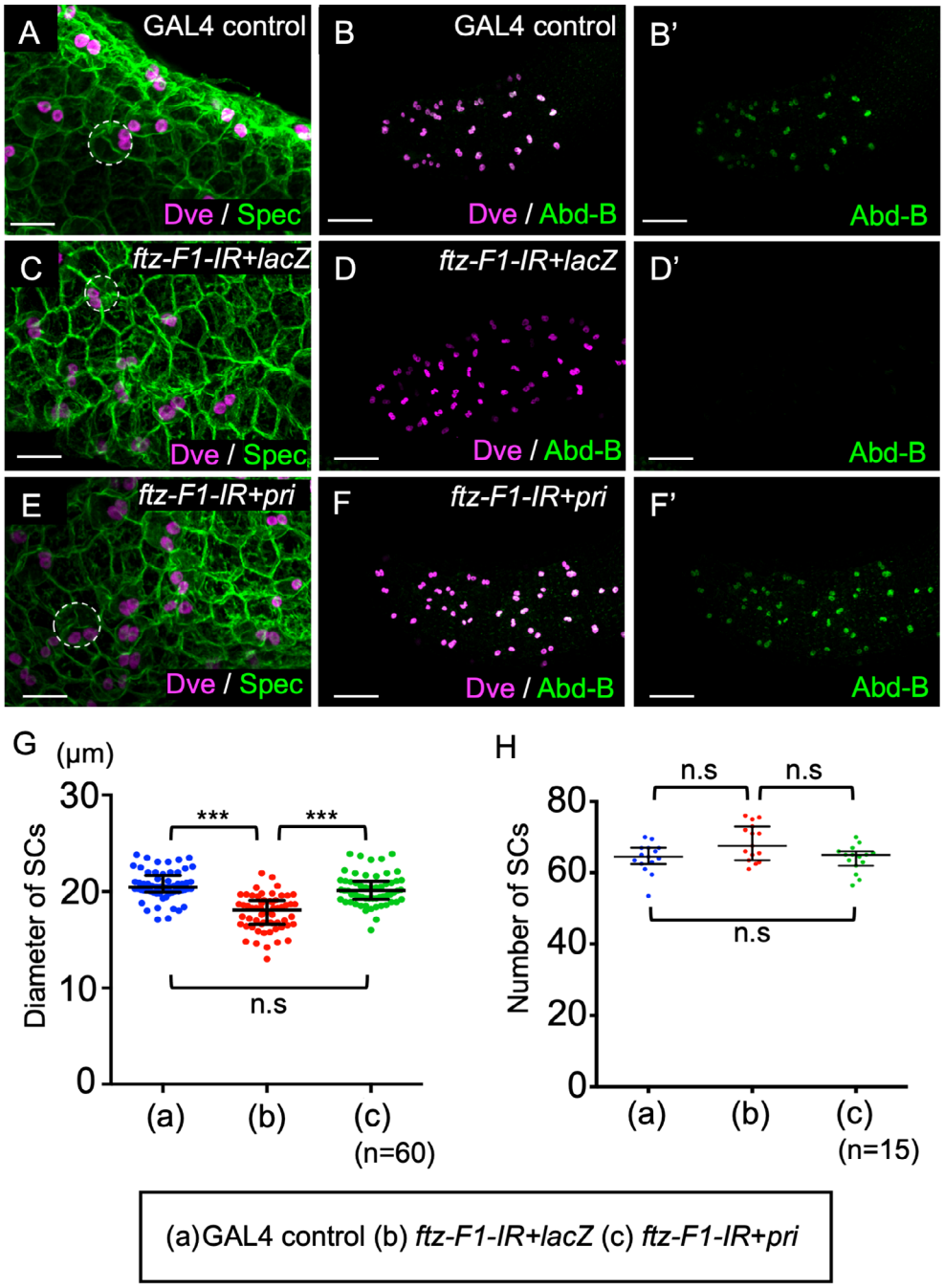

FIGURE 3 | Phenotypes of *ftz-f1* KD are rescued with co-expression of *pri*. (A–F) Accessory glands of adult day5 males of *dG30A/+* (A, B; GAL4 control), *dG30A>UAS-ftz-F1-IR + UAS-GFP::LacZ.nls* (C, D; *ftz-F1-IR + lacZ*), and *dG30A>UAS-ftz-F1-IR + UAS-pri* (E, F; *ftz-F1-IR + pri*). Expression patterns of Spectrin and Abd-B (green), and Dve (magenta) are shown. Single channel images of Abd-B expression are shown in (B′, D′, F′). Scale bar: 20 μm (A, C, E), 50 μm (B, B′ D, D′, F, F′). (G, H) Diameter (G) and number (H) of SCs in accessory glands of the indicated genotypes. The number (n) of scored SCs (G) and accessory glands (H) is indicated, respectively.

4.2 | Immunohistochemistry

Adult day5 males were dissected in phosphate-buffered saline (PBS). Fixation was performed in 4% formaldehyde/PBS 0.3% Triton X-100 for 13 min and then washed three times with PBS-0.3% Triton X-100. The following primary antibodies were used: rabbit anti-Dve (1:1000) (Nakagoshi et al. 1998), mouse anti-Abd-B (1:20), mouse anti-α-Spectrin (3A9) (1:100) (Developmental Studies Hybridoma Bank, DSHB), and rabbit anti-cleaved Caspase-3 (1:500) (Cell Signaling Technology #9661). FITC, Cy3-, or Cy5-conjugated secondary antibodies (Jackson Immunoresearch) were used for detection. Confocal images of 1 μm sections were obtained using a confocal microscope (Olympus FV1200). Fluoview, Imaris, and Prism 6 were used for information processing.

4.3 | Quantification and Statistical Analysis

To visualize the gross morphology and cell size, half-section views of the accessory gland were generated as projection

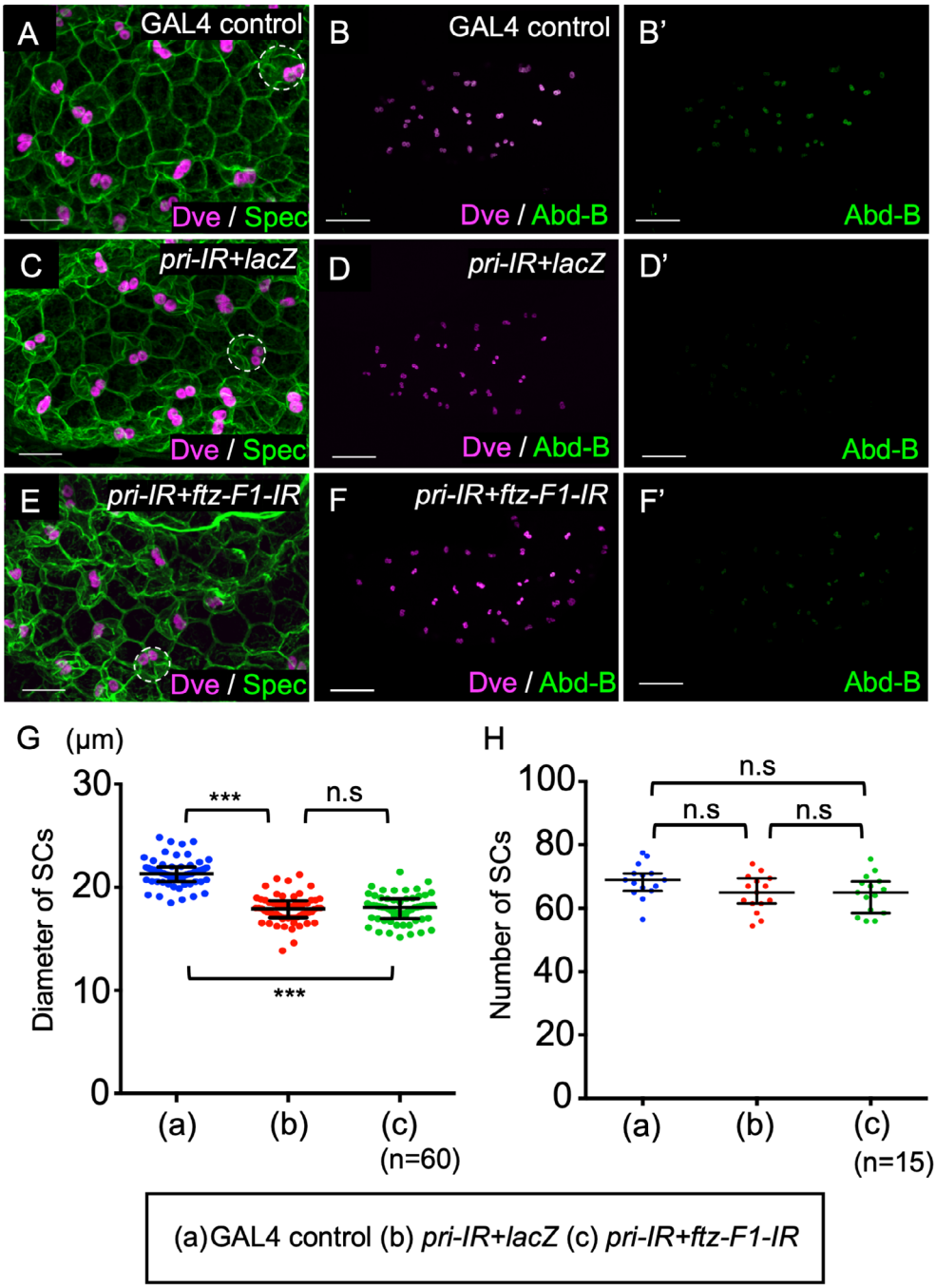

FIGURE 4 | Phenotypes of *pri* and *ftz-F1* double KD are same as *pri* KD. (A–F) Accessory glands of adult day5 males of *dG30A/+* (A, B; GAL4 control), *dG30A>UAS-pri-IR + UAS-GFP::LacZ.nls* (C, D; *pri-IR + lacZ*), and *dG30A>UAS-pri-IR + UAS-ftz-F1-IR* (E, F; *pri-IR + ftz-f1-IR*). Expression patterns of Spectrin and Abd-B (green), and Dve (magenta) are shown. Single channel images of Abd-B expression are shown in (B′, D′, F′). Scale bar: 20 μm (A, C, E), 50 μm (B, B′ D, D′, F, F′). (G, H) Diameter (G) and number (H) of SCs in accessory glands of the indicated genotypes. The number (n) of scored SCs (G) and accessory glands (H) is indicated, respectively.

images. The number of SCs was estimated from Dve-positive cells, and the spots of nuclei were calculated by Imaris software (Oxford Instruments). Diameters of round-shaped SCs and fluorescence intensity of Abd-B expression were also measured by Imaris. Data are presented as scattered plots. For each plot, the upper and bottom lines represent the first and third percentiles, and the middle line is the median.

The significance of differences between the control and test progenies was analyzed using Prism6 (GraphPad Software). For two groups comparison, Welch's *t*-test was applied to compare the means of the diameter of SCs or Abd-B expression level, and Mann–Whitney *U* test was applied to compare the number of SCs. Between multiple groups, one-way ANOVA with Holm-Sidak's multiple comparisons test was applied to compare the means of the diameter of SCs or Abd-B expression level, and Kruskal-Wallis test with Dunn's multiple comparisons test was applied to compare the number of SCs. The levels of significance are indicated by asterisks: $*p < 0.01$, $**p < 0.001$, $***$ $p < 0.0001$, n.s.: not significant.

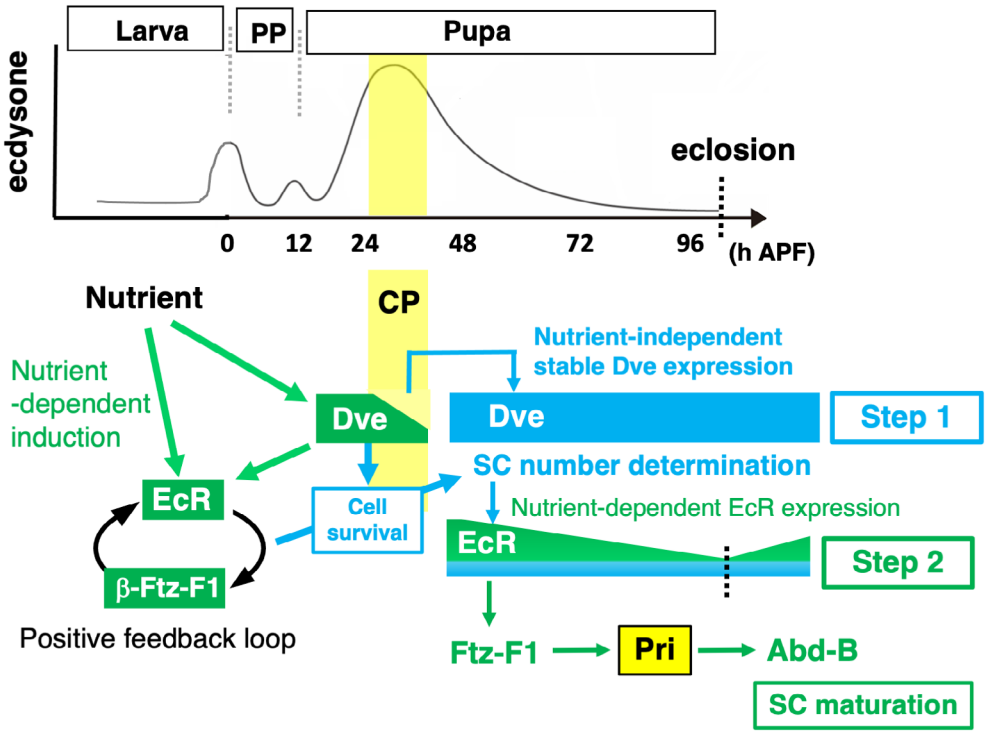

FIGURE 5 | A schematic model for Pri-mediated secondary cell maturation. In the larval stage, EcR expression is induced under nutrient-rich conditions to coordinate growth and differentiation, and Ftz-F1 is required to maintain EcR expression (positive feedback loop). From the prepupal (PP) to early-pupal stages, Dve expression is induced in a nutrient signal-dependent manner, and the expression over a threshold level in a critical period (CP; shown with yellow square) is stably maintained and becomes in a nutrient signal-independent manner. Nutrient conditions during the CP determine the level of Dve expression, namely the number of Dve^+ SCs (Step 1). EcR expression is maintained by this Dve activity in these immature SCs. In the late pupal stage, the level of EcR expression still depends on nutrient conditions. Activities of the downstream effectors Ftz-F1 and Pri optimize maturation of SCs (Step 2). The relative level of ecdysone during development is shown above.

4.4 | Progeny Assay

Male fecundity was measured as the number of viable progenies per mated wild-type female for 4 days. A group of 5 virgin females with 15 males (5-days old) was crossed in a fresh vial and allowed to lay eggs for 2 days and then transferred to a new vial for a further 2 days. The number of adult progenies from each vial was counted. The average number of progenies per mated wild-type female was calculated with 6 independent groups. A one-way ANOVA with Holm-Sidak's multiple comparisons test was applied to compare the number of progenies derived from males of each genotype.

Author Contributions

Shinichi Otsune: investigation, data curation, validation, project administration, writing – original draft. **Mirai Matsuka:** investigation. **Chisato Shirakashi:** investigation. **Xuanshuo Zhang:** data curation, writing – review and editing. **Hideki Nakagoshi:** conceptualization, writing – review and editing, resources, supervision, funding acquisition.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

References

Agawa, Y., M. Sarhan, Y. Kageyama, et al. 2007. "*Drosophila* Blimp-1 Is a Transient Transcriptional Repressor That Controls Timing of the Ecdysone-Induced Developmental Pathway." *Molecular and Cellular Biology* 27, no. 24: 8739–8747. https://doi.org/10.1128/MCB.01304-07.

Ahmad, S. M., and B. S. Baker. 2002. "Sex-Specific Deployment of FGF Signaling in *Drosophila* Recruits Mesodermal Cells Into the Male Genital Imaginal Disc." *Cell* 109, no. 5: 651–661. https://doi.org/10.1016/S0092-8674(02)00744-4.

Akagi, K., M. Sarhan, A. R. Sultan, et al. 2016. "A Biological Timer in the Fat Body Comprising Blimp-1, betaFtz-f1 and Shade Regulates Pupation Timing in *Drosophila Melanogaster*." *Development* 143, no. 13: 2410–2416. https://doi.org/10.1242/dev.133595.

Albuquerque, J. P., V. Tobias-Santos, A. C. Rodrigues, F. B. Mury, and R. N. da Fonseca. 2015. "Small ORFs: A New Class of Essential Genes for Development." *Genetics and Molecular Biology* 38, no. 3: 278–283. https://doi.org/10.1590/S1415-475738320150009.

Chanut-Delalande, H., Y. Hashimoto, A. Pelissier-Monier, et al. 2014. "Pri Peptides Are Mediators of Ecdysone for the Temporal Control of Development." *Nature Cell Biology* 16, no. 11: 1035–1044. https://doi.org/10.1038/ncb3052.

Corrigan, L., S. Redhai, A. Leiblich, et al. 2014. "BMP-Regulated Exosomes From *Drosophila* Male Reproductive Glands Reprogram Female Behavior." *Journal of Cell Biology* 206, no. 5: 671–688. https://doi.org/10.1083/jcb.201401072.

Deroo, B. J., and K. S. Korach. 2006. "Estrogen Receptors and Human Disease." *Journal of Clinical Investigation* 116, no. 3: 561–570. https://doi.org/10.1172/JCI27987.

Faunes, F., and J. Larrain. 2016. "Conservation in the Involvement of Heterochronic Genes and Hormones During Developmental Transitions." *Developmental Biology* 416, no. 1: 3–17. https://doi.org/10.1016/j.ydbio.2016.06.013.

Galindo, M. I., J. I. Pueyo, S. Fouix, S. A. Bishop, and J. P. Couso. 2007. "Peptides Encoded by Short ORFs Control Development and Define a New Eukaryotic Gene Family." *PLoS Biology* 5, no. 5: e106. https://doi.org/10.1371/journal.pbio.0050106.

Gallois, M., D. Menoret, S. Marques-Prieto, et al. 2024. "Pri Peptides Temporally Coordinate Transcriptional Programs During Epidermal Differentiation." *Science Advances* 10, no. 6: eadg8816. https://doi.org/10.1126/sciadv.adg8816.

Gligorov, D., J. L. Sitnik, R. K. Maeda, M. F. Wolfner, and F. Karch. 2013. "A Novel Function for the Hox Gene *Abd-B* in the Male Accessory Gland Regulates the Long-Term Female Post-Mating Response in *Drosophila*." *PLoS Genetics* 9, no. 3: e1003395. https://doi.org/10.1371/journal.pgen.1003395.

Hentze, J. L., M. E. Moeller, A. F. Jorgensen, et al. 2013. "Accessory Gland as a Site for Prothoracicotropic Hormone Controlled Ecdysone Synthesis in Adult Male Insects." *PLoS One* 8, no. 2: e55131. https://doi.org/10.1371/journal.pone.0055131.

Huang, X., J. T. Warren, and L. I. Gilbert. 2008. "New Players in the Regulation of Ecdysone Biosynthesis." *Journal of Genetics and Genomics* 35, no. 1: 1–10. https://doi.org/10.1016/S1673-8527(08)60001-6.

Johnston, R. J., Jr., Y. Otake, P. Sood, et al. 2011. "Interlocked Feedforward Loops Control Cell-Type-Specific Rhodopsin Expression in the *Drosophila* Eye." *Cell* 145, no. 6: 956–968. https://doi.org/10.1016/J.Cell.2011.05.003.

Khitun, A., T. J. Ness, and S. A. Slavoff. 2019. "Small Open Reading Frames and Cellular Stress Responses." *Molecular Omics* 15, no. 2: 108–116. https://doi.org/10.1039/c8mo00283e.

Kitamata, M., Y. Otake, H. Kitagori, et al. 2024. "Functional Opsin Patterning for *Drosophila* Color Vision Is Established Through Signaling Pathways in Adjacent Object-Detection Neurons." *Development* 151, no. 6: dev202388. https://doi.org/10.1242/dev.202388.

Kondo, T., Y. Hashimoto, K. Kato, S. Inagaki, S. Hayashi, and Y. Kageyama. 2007. "Small Peptide Regulators of Actin-Based Cell Morphogenesis Encoded by a Polycistronic mRNA." *Nature Cell Biology* 9, no. 6: 660–665. https://doi.org/10.1038/ncb1595.

Kondo, T., S. Plaza, J. Zanet, et al. 2010. "Small Peptides Switch the Transcriptional Activity of Shavenbaby During *Drosophila* Embryogenesis." *Science* 329, no. 5989: 336–339. https://doi.org/10.1126/science.1188158.

Kubo, A., M. Matsuka, R. Minami, et al. 2018. "Nutrient Conditions Sensed by the Reproductive Organ During Development Optimize Male Fecundity in *Drosophila*." *Genes to Cells* 23, no. 7: 557–567. https://doi.org/10.1111/gtc.12600.

Lavorgna, G., F. D. Karim, C. S. Thummel, and C. Wu. 1993. "Potential Role for a FTZ-F1 Steroid Receptor Superfamily Member in the Control of *Drosophila* Metamorphosis." *Proceedings of the National Academy of Sciences of the United States of America* 90, no. 7: 3004–3008. https://doi.org/10.1073/pnas.90.7.3004.

Leiblich, A., J. Hellberg, A. Sekar, et al. 2019. "Mating Induces Switch From Hormone-Dependent to Hormone-Independent Steroid Receptor-Mediated Growth in *Drosophila* Secondary Cells." *PLoS Biology* 17, no. 10: e3000145. https://doi.org/10.1371/journal.pbio.3000145.

Mani, S. K., P. G. Mermelstein, M. J. Tetel, and G. Anesetti. 2012. "Convergence of Multiple Mechanisms of Steroid Hormone Action." *Hormone and Metabolic Research* 44, no. 8: 569–576. https://doi.org/10.1055/s-0032-1306343.

Matsuka, M., and H. Nakagoshi. 2019. "Nutrient Conditions Optimize Male Fecundity in *Drosophila Melanogaster*." In *Advances in Medicine and Biology*, edited by L. V. Berhardt, vol. 141, 191–211. Nova Science Publishers.

Matsuka, M., S. Otsune, S. Sugimori, Y. Tsugita, H. Ueda, and H. Nakagoshi. 2024. "Fecundity Is Optimized by Levels of Nutrient Signal-Dependent Expression of Dve and EcR in *Drosophila* Male Accessory Gland." *Developmental Biology* 508: 8–23. https://doi.org/10.1016/j.ydbio.2024.01.004.

Minami, R., M. Wakabayashi, S. Sugimori, et al. 2012. "The Homeodomain Protein Defective Proventriculus Is Essential for Male Accessory Gland Development to Enhance Fecundity in *Drosophila*." *PLoS One* 7, no. 3: e32302. https://doi.org/10.1371/journal.pone.0032302.

Moeller, M. E., S. Nagy, S. U. Gerlach, et al. 2017. "Warts Signaling Controls Organ and Body Growth Through Regulation of Ecdysone." *Current Biology* 27, no. 11: 1652–1659. https://doi.org/10.1016/j.cub.2017.04.048.

Nakagawa, Y., S. Fujiwara-Fukuta, T. Yorimitsu, et al. 2011. "Spatial and Temporal Requirement of Defective Proventriculus Activity During *Drosophila* Midgut Development." *Mechanisms of Development* 128: 258–267. https://doi.org/10.1016/j.mod.2011.02.003.

Nakagoshi, H., M. Hoshi, Y. Nabeshima, and F. Matsuzaki. 1998. "A Novel Homeobox Gene Mediates the Dpp Signal to Establish Functional Specificity Within Target Cells." *Genes & Development* 12, no. 17: 2724–2734. https://doi.org/10.1101/gad.12.17.2724.

Nakagoshi, H., T. Shirai, Y. Nabeshima, and F. Matsuzaki. 2002. "Refinement of Wingless Expression by a Wingless- and Notch-Responsive Homeodomain Protein, Defective Proventriculus." *Development (Cambridge, England)* 249, no. 1: 44–56. https://doi.org/10.1006/dbio.2002.0746.

Ohno, C. K., and M. Petkovich. 1993. "FTZ-F1 Beta, a Novel Member of the *Drosophila* Nuclear Receptor Family." *Mechanisms of Development* 40, no. 1–2: 13–24. https://doi.org/10.1016/0925-4773(93)90084-b.

Ou, Q., and K. King-Jones. 2013. "What Goes Up Must Come Down: Transcription Factors Have Their Say in Making Ecdysone Pulses." *Current Topics in Developmental Biology* 103: 35–71. https://doi.org/10.1016/B978-0-12-385979-2.00002-2.

Petryk, A., J. T. Warren, G. Marques, et al. 2003. "Shade Is the *Drosophila* P450 Enzyme That Mediates the Hydroxylation of Ecdysone to the Steroid Insect Molting Hormone 20-Hydroxyecdysone." *Proceedings of the National Academy of Sciences of the United States of America* 100, no. 24: 13773–13778. https://doi.org/10.1073/pnas.2336088100.

Pi, H., Y. C. Huang, I. C. Chen, C. D. Lin, H. F. Yeh, and L. M. Pai. 2011. "Identification of 11-Amino Acid Peptides That Disrupt Notch-Mediated Processes in *Drosophila*." *Journal of Biomedical Science* 18, no. 1: 42. https://doi.org/10.1186/1423-0127-18-42.

Plaza, S., G. Menschaert, and F. Payre. 2017. "In Search of Lost Small Peptides." *Annual Review of Cell and Developmental Biology* 33: 391–416. https://doi.org/10.1146/annurev-cellbio-100616-060516.

Pueyo, J. I., E. G. Magny, and J. P. Couso. 2016. "New Peptides Under the s(ORF)ace of the Genome." *Trends in Biochemical Sciences* 41, no. 8: 665–678. https://doi.org/10.1016/j.tibs.2016.05.003.

Ray, S., M. I. Rosenberg, H. Chanut-Delalande, et al. 2019. "The Mlpt/Ubr3/Svb Module Comprises an Ancient Developmental Switch for Embryonic Patterning." *eLife* 8: e39748. https://doi.org/10.7554/eLife.39748.

Redhai, S., J. E. Hellberg, M. Wainwright, et al. 2016. "Regulation of Dense-Core Granule Replenishment by Autocrine BMP Signalling in *Drosophila* Secondary Cells." *PLoS Genetics* 12, no. 10: e1006366. https://doi.org/10.1371/journal.pgen.1006366.

Rewitz, K. F., N. Yamanaka, and M. B. O'Connor 2013. "Developmental Checkpoints and Feedback Circuits Time Insect Maturation." *Current Topics in Developmental Biology* 103: 1–33. https://doi.org/10.1016/B978-0-12-385979-2.00001-0.

Saghatelian, A., and J. P. Couso. 2015. "Discovery and Characterization of smORF-Encoded Bioactive Polypeptides." *Nature Chemical Biology* 11, no. 12: 909–916. https://doi.org/10.1038/nchembio.1964.

Sharma, V., A. K. Pandey, A. Kumar, et al. 2017. "Functional Male Accessory Glands and Fertility in *Drosophila* Require Novel Ecdysone Receptor." *PLoS Genetics* 13, no. 5: e1006788. https://doi.org/10.1371/journal.pgen.1006788.

Shirai, T., T. Yorimitsu, N. Kiritooshi, F. Matsuzaki, and H. Nakagoshi. 2007. "Notch Signaling Relieves the Joint-Suppressive Activity of Defective Proventriculus in the Drosophila Leg." *Developmental Biology* 312, no. 1: 147–156. https://doi.org/10.1016/j.ydbio.2007.09.003.

Sultan, A. R. S., Y. Oish, and H. Ueda. 2014. "Function of the Nuclear Receptor FTZ-F1 During the Pupal Stage in *Drosophila melanogaster*." *Development, Growth & Differentiation* 56, no. 3: 245–253. https://doi.org/10.1111/dgd.12125.

Taira, Y., H. Wada, S. Hayashi, and Y. Kageyama. 2021. "Polished Rice Mediates Ecdysone-Dependent Control of *Drosophila* Embryonic Organogenesis." *Genes to Cells* 26, no. 5: 269–281. https://doi.org/10.1111/gtc.12841.

Taniguchi, K., A. Kokuryo, T. Imano, R. Minami, H. Nakagoshi, and T. Adachi-Yamada. 2012. "Binucleation of *Drosophila* Adult Male Accessory Gland Cells Increases Plasticity of Organ Size for Effective Reproduction." *Bio Systems* 1: e101. https://doi.org/10.4172/2329-6577.1000e101.

Taniguchi, K., A. Kokuryo, T. Imano, H. Nakagoshi, and T. Adachi-Yamada. 2018. "Binucleation of Accessory Gland Lobe Contributes to Effective Ejection of Seminal Fluid in *Drosophila melanogaster*." *Zoological Science* 35, no. 5: 446–458. https://doi.org/10.2108/zs170188.

Yorimitsu, T., N. Kiritooshi, and H. Nakagoshi. 2011. "Defective Proventriculus Specifies the Ocellar Region in the *Drosophila* Head." *Developmental Biology* 356: 598–607. https://doi.org/10.1016/j.ydbio.2011.06.015.

Yussa, M., U. Lohr, K. Su, and L. Pick. 2001. "The Nuclear Receptor Ftz-F1 and Homeodomain Protein Ftz Interact Through Evolutionarily Conserved Protein Domains." *Mechanisms of Development* 107, no. 1–2: 39–53. https://doi.org/10.1016/s0925-4773(01)00448-8.

Zanet, J., E. Benrabah, T. Li, et al. 2015. "Pri sORF Peptides Induce Selective Proteasome-Mediated Protein Processing." *Science* 349, no. 6254: 1356–1358. https://doi.org/10.1126/science.aac5677.

Supporting Information

Additional supporting information can be found online in the Supporting Information section.