The Homeodomain Protein Defective Proventriculus Is Essential for Male Accessory Gland Development to Enhance Fecundity in Drosophila

Ryunosuke Minami¹, Miyuki Wakabayashi¹, Seiko Sugimori¹, Kichiro Taniguchi², Akihiko Kokuryo³,⁴, Takao Imano³,⁴, Takashi Adachi-Yamada²,³,⁴, Naoko Watanabe⁵, Hideki Nakagoshi¹*

¹ Graduate School of Natural Science and Technology, Okayama University, Okayama, Japan, ² Department of Life Science, Faculty of Science, Gakushuin University, Tokyo, Japan, ³ Institute of Biomolecular Science, Gakushuin University, Tokyo, Japan, ⁴ Department of Biology, Graduate School of Science, Kobe University, Kobe, Japan, ⁵ Department of Biomolecular Science, Faculty of Science, Toho University, Funabashi, Japan

Abstract

The Drosophila male accessory gland has functions similar to those of the mammalian prostate gland and the seminal vesicle, and secretes accessory gland proteins into the seminal fluid. Each of the two lobes of the accessory gland is composed of two types of binucleate cell: about 1,000 main cells and 40 secondary cells. A well-known accessory gland protein, sex peptide, is secreted from the main cells and induces female postmating response to increase progeny production, whereas little is known about physiological significance of the secondary cells. The homeodomain transcriptional repressor Defective proventriculus (Dve) is strongly expressed in adult secondary cells, and its mutation resulted in loss of secondary cells, mononucleation of main cells, and reduced size of the accessory gland. dve mutant males had low fecundity despite the presence of sex peptide, and failed to induce the female postmating responses of increased egg laying and reduced sexual receptivity. RNAi-mediated dve knockdown males also had low fecundity with normally binucleate main cells. We provide the first evidence that secondary cells are crucial for male fecundity, and also that Dve activity is required for survival of the secondary cells. These findings provide new insights into a mechanism of fertility/fecundity.

Introduction

In many higher insects, the reproductive behavior of females drastically changes after mating [1,2]. Stimulation of egg laying and suppression of remating are induced by factors present in the male seminal fluid. The Drosophila male accessory gland secretes accessory gland proteins (Acps) into the seminal fluid, which are essential for male fertility/fecundity [3,4]. Each of the two lobes of the accessory gland is composed of two types of binucleate cell: about 1,000 main cells and 40 secondary cells [5]. Adult main cells are flat hexagonal cells and secondary cells are large spherical cells interspersed among the main cells at the distal tip of each accessory gland lobe. A well-known Acp, sex peptide (SP, also known as Acp70A), is secreted from the main cells and induces long-term postmating response, such as increased egg laying and reduced sexual receptivity, to increase progeny production [6,7,8,9,10]. These postmating responses are critically regulated through SP binding to the G-protein-coupled SP receptor in the female reproductive tract [11,12,13]. In addition, SP-sperm interaction is also required for long-term postmating response through localization of SP to sperm storage organs, and the C-terminal part of SP is gradually released from sperm tails [14,15]. In contrast to the increasing knowledge of Acps secreted from the main cells, little is known about physiological significance of the secondary cells.

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 disk during late larval development [16]. The btl-expressing cells become epithelial, and give rise to accessory glands (paragonia) and seminal vesicles (vas deferens). Subsequent cell proliferation and functional differentiation of the accessory gland are regulated by the homeodomain transcription factor Paired (Prl) [17]. Mutant males for prl are sterile as they have severely reduced or no accessory glands [18,19,20], indicating that seminal fluid components from the accessory gland are essential for male fertility.

The homeodomain transcriptional repressor Defective proventriculus (Dve) is involved in various functions including wing morphogenesis, leg joint formation, head vertex specification, ommatidial cell-type specification, and functional specification of the midgut [21,22,23,24,25,26,27]. Here, we provide evidence that Dve is required for male accessory gland development, binucleation of main cells and survival of secondary cells, and also that secondary cells are essential for male fecundity.

Citation: Minami R, Wakabayashi M, Sugimori S, Taniguchi K, Kokuryo A, et al. (2012) The Homeodomain Protein Defective Proventriculus Is Essential for Male Accessory Gland Development to Enhance Fecundity in Drosophila. PLoS ONE 7(3): e32302. doi:10.1371/journal.pone.0032302

Editor: Barbara Jennings, University College London, United Kingdom

Received November 18, 2011; Accepted January 26, 2012; Published March 12, 2012

Copyright: © 2012 Minami et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This work was partly supported by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (20570202 to TAY, 22019034 to KT). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. No additional external funding was received for this study.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: goshi@cc.okayama-u.ac.jp
Results

Spatio-temporal pattern of Dve expression during accessory gland development

We have found that Dve is expressed in the male accessory gland at least from 24 hr after puparium formation (APF) but not in the male primordia of the genital disc in the late third-larval instar (Figure 1A, 1B). Dve is expressed strongly in secondary cells and weakly in main cells at 72 hr APF (Figure 1C), but Dve expression in main cells is undetectable in the adult stage (Figure 1D). To generate a GAL4 driver line that can induce gene expression during early stages of accessory gland development, we established a dve-GAL4 line, in which GAL4 expression is under the control of the 13-kb regulatory element upstream of the first exon of dve. Expression pattern of the green fluorescent protein (GFP) driven by the dve-GAL4[35A] (dG35A.GFP) is nearly identical to that of endogenous Dve protein, although low-level expression in main cells could be detected in the adult stage (Figure 1E–H).

The homeobox gene paired (prd) is required for accessory gland development and its mutation leads to greatly reduced size of the accessory gland [17]. We compared Dve expression with that of Prd, and found that the onset of their expression was nearly the same (Figure 1I–K). However, Prd expression in main cells is maintained in the adult stage (Figure 1I). In the midgut, Dve is coexpressed with the copper cell determinant Labial in embryonic precursor cells, and the subsequent dve repression in the larval copper cells is regulated by Labial and Dve itself [24]. We hypothesized that the temporally regulated dve repression in accessory gland main cells is also regulated in a similar manner. Therefore, we examined the effect of prd mutation on Dve expression. Interestingly, Dve was derepressed in prd mutant main cells, which also had lost their binucleate character (Figure 1M). In contrast, prd mutant secondary cells were normally binucleate and Dve expression was maintained in these cells (Figure 1N). Thus, the Prd activity is required for temporally regulated dve repression in the main cells and it is dispensable for Dve expression in the secondary cells.

Dve-A is required for secondary cell development

Two transcripts, type A (4.9 kb) and type B (3.5 kb), have been identified in the dve locus. The type-A (dve-A) null allele dveE181 has about 4-kb deletion including the first exon for the dve-A transcript, while the dveE144 allele has a smaller deletion [22]. Homozygosity

![Figure 1. Dve expression during accessory gland development.](image-url)
for *dve* 	extsuperscript{E181} is semi-lethal, and escaper adult males have smaller accessory glands (Figure 2A, 2B). Because the body size of *dve* 	extsuperscript{E181} homozygous males was also small (Figure 2D), we measured the size of ejaculatory bulb as an internal control. The size ratio of cross-section area (accessory gland/ejaculatory bulb, AG/EB) was significantly reduced in males of *dve* 	extsuperscript{E181} homozygotes and heteroallelic combination (*dve* 	extsuperscript{E181}/*dve* 	extsuperscript{E144}) (Figure 2E). Similar phenotype was also observed in RNAi-mediated *dve* knockdown (*dve* KD, dG30A>dve-IR *dve* 	extsuperscript{1}) (Figure 2E and Figure S1). To perform efficient knockdown, we recombined *UAS-dve-IR* with a loss of function allele, *dve* 	extsuperscript{1} [24,25], to generate a recombinant chromosome, *UAS-dve-IR dve* 	extsuperscript{1}. Notably, these *dve*-A mutant accessory glands completely lacked cells with the appearance of mature secondary cells and some of their main cells were mononucleate (Figure 2B', 2B'', 2F). Secondary cells can be distinguished by their spherical cell shape and the presence of large vacuoles. The vacuolar components are non-specifically stained with anti-Prd antibody (red in Figure 2A', 2A''). Interestingly, they were rarely detected in very small atrophic cells of *dve*-A mutants (arrow in Figure 2B''), suggesting that cell fate for the secondary cell could be induced in *dve*-A mutants.

Because *prd* mutant main cells are mononucleate (Figure 1M), we checked whether these *dve*-A mutant phenotypes are due to the absence of Prd activity. Prd expression was unaffected in *dve* 	extsuperscript{E181} mutants (Figure 2B''), indicating that the Dve activity is crucial for growth of the accessory gland, binucleation of main cells, and secondary cell differentiation even in the presence of Prd proteins.

To further confirm the Dve functions in accessory gland

![Figure 2. Dve-A is required for secondary cell development.](image)

**Figure 2. Dve-A is required for secondary cell development.** (A and B) Reproductive organs of a control male (A: *dve* 	extsuperscript{E181}/CyOGFP) and a *dve*-A mutant male (B: *dve* 	extsuperscript{E181} homozygote). The size of mutant accessory glands (AG, arrows) is small compared with the size of ejaculatory bulb (EB, arrowheads). Scale bar is 200 μm. A control AG has about 20 secondary cells in a half-plane (A' and A''), and the cytoplasmic vacuolar components are non-specifically stained with rabbit anti-Prd antibody (red in A''). Nuclei (DNA) and cell membrane are labeled with TO-PRO3 (blue) and anti-Spectrin antibody (green), respectively. Binucleate Prd expression is shown in magenta (A' and A''). A *dve*-A mutant AG has mononucleate main cells that normally express Prd (magenta in B' and B''). The *dve*-A mutant AG has no mature secondary cells, although the signal for vacuolar components (red) is rarely detected in a very small atrophic cell (arrow in B''). (C and C') A *dve*-A mutant mosaic AG of 4–6 days old adult. Mutant clones are marked by the absence of GFP expression (green) and are outlined in C'. No secondary cells are detected within *dve*-mutant clones (C), and mononucleate cells are detected only in mutant clones (C'). (D–F) Comparison of body mass (D), size ratio of AG/EB (E), and rate of mononucleate cells (MNC) (F) between males of the indicated genotypes. N indicates the number of individuals tested. Error bars show standard error of means (SEM) (** p<0.001, * P<0.01, t-test). RNAi-mediated *dve* knockdown (*dve* KD: dG30A>dve-IR *dve* 	extsuperscript{1}) also shows reduced AGs, which have relatively normal binucleation compared with those of *dve*-A mutants.

doi:10.1371/journal.pone.0032302.g002
development, we performed mosaic analysis using the loss of function allele dve1. Mutant clones for dve1 showed the same phenotypes as the accessory gland did in a dve-A mutant male. Cells with secondary morphology were only detected in GFP-positive wild-type cells but not within dve1 mutant clones (Figure 2C, 2C’).

Seminal fluid from dve mutant males is defective despite the presence of sex peptide

As prev mutant males who have very small accessory glands are sterile due to a defect in ejaculation [18, 19, 20], we checked the fertility/fecundity of dve911 mutant males. The dve-A mutant sperms had normal motility and they were correctly localized to the female sperm storage organs, seminal receptacle (src) and spermatheca (sp) (Figure 3A-G). However, the average number of progeny from a wild-type female mated with a dve-A mutant male was significantly lower than those mated with control males (Figure 4A). Therefore, it is assumed that the low fecundity of dve-A mutant males reflects the defects of seminal fluid proteins that are secreted from accessory gland cells. We examined two potential reasons for the low fecundity: (1) failure of egg-laying stimulation, and (2) reduced rate of fertilization as reported in wasted mutant sperms [28].

Females mated with dve-A mutant males laid substantially fewer eggs than those mated with control males, although the hatching rate was indistinguishable between females mated with dve-A mutant or control males (Figure 4B and data not shown). These results strongly suggest that the low fecundity of dve-A mutant males is due to the failure of egg-laying stimulation to mated females.

The sex peptide (SP), also known as Acp70A, is a major accessory gland protein that induces changes in female postmating response such as egg laying and receptivity [7, 10]. Females mated with SP null mutant males lay fewer eggs, and they show high receptivity to the second males (i.e., remating activity) at least 12 hr after mating. The mating plug protein PEBII, which is secreted from the ejaculatory bulb, contributes to reduced receptivity at earlier period (4 hr after mating) [29]. Thus, we examined remating activity of a female firstly mated with a dve-A mutant male and secondly with a wild-type male. The remating activity at 4 hr after first mating was not evident (Figure 4C).

Interestingly, females at 40 hr after first mating with dve-A mutant males showed high remating activity (Figure 4D). These results raised the possibility that SP expression or its transfer is impaired in dve-A mutant males. Thus, the SP expression in main cells was monitored using a GFP fusion protein (SP::GFP), whose expression is under the control of the endogenous SP promoter [30]. Unexpectedly, SP::GFP was normally expressed and secreted into the lumen of the dve-A mutant accessory glands, and it was normally transferred into the female reproductive tract after mating (Figure 3H-J).

Secondary cells are essential for male fecundity

To examine whether the low fecundity of dve mutant males is due to the mononucleate state of SP-expressing main cells, we checked the postmating response of females after they had mated with dve KD males. In dve KD males, binucleation of main cells was relatively normal in contrast to mononucleate main cells seen in dve-A mutants (Figure 2F and Figure 5A). This does not mean that dve KD main cells are functionally normal, however, secondary cell differentiation was severely inhibited and small atrophic cells were frequently observed (Figure 5A’). Females mated with the dve KD or dve-A mutant males showed the same phenotypes in egg laying and remating activity (Figure 5B-D), suggesting the importance of mature secondary cells for male fecundity.

Although little is known about physiological significance of secondary cells, Abd-B mutant males also lack mature secondary cells and show low fecundity similar to that of dve-A mutants (personal communication from M. F. Wolfner and F. Karch). These results further support the importance of secondary cells and prompted us to examine the relationship between Dve and Abd-B during secondary cell development. Expression patterns of Dve and Abd-B are nearly complementary until 24 hr APF and change to the same pattern from 48 hr APF to the adult stage (Figure S2A–H). Abd-B expression after the late pupal stage appears to be dependent on the Dve activity, because it was greatly reduced in dve mutant clones and also in adjacent wild-type cells (Figure S2E). Thus, it seems likely that the low fecundity in dve mutant males reflects the absence of Abd-B expression with loss of mature secondary cells. Taken together, these results strongly suggest that unknown factors secreted from secondary cells are essential for male fecundity.

The common region of Dve isoforms is required for survival of secondary cells

In dve-A mutant or dve KD accessory glands, we could detect very small atrophic cells (Figure 2B’ and Figure 5A’). These observations suggest a mechanism that Dve is required for cell survival rather than cell-fate determination of secondary cells. Consistent with this notion, we could detect a few secondary cell precursors in dve-A mutants during pupal development. These mutant cells had a characteristic feature of secondary cells in their nuclear arrangement and also showed reduced Prd expression (Figure 6A, 6B). Because Prd expression in main cells was unaffected in dve mutants (Figure 2B’, 2C and Figure S2E), these cells appear to be precursors of atrophic secondary cells and they were detected only in the distal region (Figure 6B). It seems likely that most precursor cells rapidly die in dve-A mutants, because it was quite difficult to detect these precursor cells even in earlier stages.

Figure 3. Localization of sperm and SP derived from dve mutant males. (A) Female reproductive organs, uterus (ut), seminal receptacle (src), and spermatheca (spt), are visualized with DNA staining (magenta) at 30 min after mating. The Protamine B-GFP fusion proteins (ProB::GFP, green) are localized in the sperm nuclei. (B–G) Localization of control sperms (B–D) and dve-A mutant sperms (E–G) in reproductive organs of wild-type females at 30 min after mating. The dve-A mutant sperms are correctly localized to the sperm storage organs (src and spt). (H–J) Localization of the sex peptide-GFP fusion proteins (SP::GFP, green) in reproductive organs of wild-type females at 20–30 min after mating. Females mated with control (H), dve-A mutant (I), and dve KD (J) males are shown. Arrows indicate the sperm mass and arrowheads indicate the posterior mating plugs.

doi:10.1371/journal.pone.0032302.g003
Loss of secondary cells in \textit{dve-A} mutants was rescued by \textit{dve-A} expression (Figure 6C). Interestingly, expression of \textit{dve-B} also rescued loss of secondary cells in the \textit{dve-A} mutant background (Figure 6D). These results imply that the common region of two isoforms including a compass domain (CMP) and two homeodomains (HD-N and HD-C) is responsible for survival of secondary cells.

**Discussion**

Mutant males for \textit{dve-A} showed low fecundity together with loss of secondary cells and reduced size of accessory glands. It has been reported that greatly reduced size of accessory glands results in sterility, and also suggested that there is a minimum size to maintain fertility [31]. If a male is selected for larger size of accessory glands with 16 generations, the selected males have good fecundity. However, they have only about 1.4-fold larger size compared to the control males, suggesting that there is also a maximal size of accessory gland not to waste energy [31]. Thus, the size of accessory glands should be controlled in an appropriate range and binucleation seems to be the best strategy to provide highly plastic change of the size [32]. Although reduced size of \textit{dve-A} mutant accessory glands may have some effects on fecundity, \textit{dve} KD males had similar size of accessory glands to the \textit{dve-A} heterozygous controls and \textit{dve} KD males showed low fecundity with loss of secondary cells (Figure 2E and Figure 5). Thus, it is most likely that the low fecundity in \textit{dve-A} mutant males is due to the absence of mature secondary cells (Figure 7). This is consistent with independent findings that Abc-B is required for maturation of secondary cells and for maintaining female postmating response (personal communication from M. F. Wolfner and F. Karch). Although we cannot exclude the possibility that some defects in \textit{dve} mutant main cells affect the fecundity, SP was normally expressed and transferred into the female reproductive tract (Figure 3H–J). Thus, the following mechanisms should be considered for SP activation to induce long-term postmating response; (1) secondary cell products cooperate in parallel with SP-mediated signaling; (2) secondary cell products enhance SP binding to its receptor; (3) secondary cell products stabilize SP binding to sperm; and (4) secondary cell products are involved in modification and/or stabilization of SP secreted from main cells. Interestingly, egg laying of females mated with \textit{dve} mutant or \textit{dve} KD males was comparable to those of virgin female controls. Error bars show SEM (** $p<0.001$, one-way ANOVA). doi:10.1371/journal.pone.0032302.g004

Figure 4. Behaviors of females mated with \textit{dve} mutant males. Postmating response of a wild-type female mated with a male of the indicated genotype. (A) The average number of progeny during 4 days. WT: wild-type male. A female mated with a \textit{dve-A} mutant male (\textit{dve}E181 or \textit{dve}E181/\textit{dve}E144) has reduced number of progeny compared with heterozygous controls. (B) The average number of eggs laid in a day. (C and D) Remating rate with a wild-type male at 4 hr (C) and 48 hr (D) after first mating with the indicated male. Mating rate of WT virgin females is also shown (black bar). Egg laying (B) and remating rate at 48 hr (D) of females mated with \textit{dve} mutant males are comparable to those of virgin female controls. Error bars show SEM (** $p<0.001$, one-way ANOVA). doi:10.1371/journal.pone.0032302.g004
gradually reduced over time (Figure 4B, 5B), suggesting that the last two interpretations, stabilization of SP by secondary cell products, are more plausible. Identification of unknown factors secreted from secondary cells will provide new insights into a mechanism that is crucial for activation of seminal fluid functions.

It seems likely that Dve functions are crucial to inhibit cell death of secondary cells, and an intriguing possibility is that inactivation of the Dve activity is closely linked to the regulated cell death to adjust the number of secondary cells. Dve and the special AT-rich sequence binding proteins (SATBs) belong to the cut superclass of homeobox genes and have an evolutionarily conserved CMP [33]. It is reported that SATB1 is cleaved and inactivated by Caspase 6 in response to the apoptotic signaling pathway [34]. Expression of the BCL2 gene, which is a key regulator to inhibit apoptosis, is

Figure 5. Behaviors of females mated with dve KD males. (A) An accessory gland in which dve is knocked down (dve KD, dG30A>dve-IR dve1). Nuclei and cell membrane are labeled with anti-Prd (magenta) and anti-Spectrin (green) antibodies, respectively. The boxed region is magnified in (A') and arrows indicate small atrophic secondary cells. (B–D) Postmating response of a wild-type female mated with a male of the indicated genotype. (B) The average number of eggs laid in a day. (C and D) Remating rate with a wild-type male at 4 hr (C) and 48 hr (D) after first mating with the indicated male. Mating rate of WT virgin females is also shown (black bar). Egg laying (B) and remating rate at 48 hr (D) of females mated with dve KD males are comparable to those of virgin female controls or females mated with SP null mutant males (SP0/D130). Error bars show SEM (** p<0.001, one-way ANOVA).
doi:10.1371/journal.pone.0032302.g005
Figure 6. Secondary cell precursors are detectable in dve mutants. (A and B) Accessory glands of a control male (A: dveE181/CyO,GFP) and a dve-A mutant male (B: dveA/dveE144) at 72 hr APF. Nuclei are labeled in magenta with anti-Prd antibody. The control secondary cell precursors (outlined in A) strongly express Abd-B (green). A dve-A mutant accessory gland has a few secondary cell precursors in which Prd expression is greatly reduced (outlined in B). (C and D) Accessory glands of 4-days old adults are labeled with anti-Prd (red) and anti-Abd-B (green) antibodies. (C) Loss of secondary cells in dve-A mutants is rescued by expressing dve-A (w; dve181/dveE144; dG30A/UAS-dveA-Y4N). (D) Loss of secondary cells in dve-A mutants is also rescued by expressing dve-B (w; dve181/dveE144; dG30A/UAS-dveB-Y4N). The rescued secondary cells are outlined. They express Abd-B (white in C and D) and have vacuole components that are non-specifically stained with anti-Prd antibody (red in C’ and D’). doi:10.1371/journal.pone.0032302.g006

finely tuned by a variety of stimuli and activated through SATB1-mediated chromatin looping. Thus, SATB1 is required for cell survival through inhibition of programmed cell death [35,36]. The functional similarity between Dve and SATB1 for inhibition of cell death raises a possibility that an evolutionarily conserved CMP plays important roles to inhibit cell death. The CMP of SATB1 is finely tuned by a variety of stimuli and activated through SATB1-mediated chromatin looping. Thus, SATB1 is required for cell survival through inhibition of programmed cell death [35,36]. The functional similarity between Dve and SATB1 for inhibition of cell death raises a possibility that an evolutionarily conserved CMP plays important roles to inhibit cell death. The CMP of SATB1 is characterized as a PDZ-like domain (amino acids 90 to 204) involved in protein-protein interaction, and the Caspase 6-characterized as a PDZ-like domain (amino acids 90 to 204) plays important roles to inhibit cell death. The CMP of SATB1 is characterized as a PDZ-like domain (amino acids 90 to 204) involved in protein-protein interaction, and the Caspase 6-mediated chromatin looping. Thus, SATB1 is required for cell survival through inhibition of programmed cell death [35,36].

Figure 7. Schematic diagram of Dve functions during accessory gland development. Dve is strongly expressed in adult secondary cells (magenta) and undetectable in adult main cells (green). The main cells secrete sex peptide that is essential for long-term postmating response to increase progeny production. The Dve activity is required for proper development of these cells: survival of the secondary cells and binucleation of the main cells. An unknown factor X secreted from the secondary cells is essential for increasing progeny production, i.e., high fecundity.

Establishment of dve-GAL4 lines

The lacZ sequence of pCaSpeR-AUG-β-gal vector [39] was replaced with the GALA sequence from pGaTB [40]. This pCaSpeR-GAL4 vector has unique restriction enzyme sites (kpnI, EcoRV, and BamHI) upstream of the GALA open reading frame. The 5.8-kb fragment, which includes the upstream promoter region and the first exon (to +557) of the dve gene, was inserted into the kpnI-BamHI sites of pCaSpeR-GAL4 to generate pCaSpeR-5.8EgA-GAL4. The 5.4-kb fragment, which includes further upstream sequences, was inserted into the kpnI-EcoRI sites of pCaSpeR-5.8EgA-GAL4 to generate pCaSpeR-5.4-5.8EgA-GAL4. The 1.6-kb EcoRI fragment was inserted into the junctional EcoRI site between 5.4-kb and 5.8-kb fragments of pCaSpeR-5.4-5.8EgA-GAL4 to generate the dve-13kb-GAL4. This construct expresses the dimerization of SATB1. Further characterization of CMP-interacting proteins will clarify the underlying mechanism and provide new insights into a regulatory mechanism of fecundity/fertility.

Materials and Methods

Fly stocks

Flies were reared on standard yeast-glucose-cornmeal-agar medium at 25°C. Oregon-R flies were used as wild-type controls. dveE181 and dveE144 are dve-A-specific mutant alleles that remove the first exon [22]. dve9 is a severe loss of function allele that has no dve-A and a very weak dve-B activity in the larval midgut [22,24]. dve1106 is a null allele that completely removes the gene [37]. To knockdown the dve activity in a spatio-temporal manner, we used UAS-dve-IR (v109538, Vienna Drosophila RNAi Center), which can induce RNA interference by expressing the inverted repeat (IR) sequence of exon 3. To perform efficient knockdown, a recombinant line, UAS-dve-IR dve9, was used for experiments. SPnull mutant males were produced by crossing SP+/TM3 Sb females with A130/TM3 Sb males. The resulting SP+/A130 males produce no SP [10]. The following GFP marker strains were used: protamineB::GFP for sperm nuclei (Drosophila Genetic Resource Center, DGRC) [38] and SP::GFP for expression of sex peptide [30].

The following GALA/UAS lines were used: btl-GALA, UAS-flp, UAS-GFP.nls (Bloomington), UAS-GFP-N-lacZ (DGRC), UAS-dveA-9B2, and UAS-Flag-dveB-Y4N (on the third chromosome) [22,24].

Establishment of dve-GAL4 lines

The lacZ sequence of pCaSpeR-AUG-β-gal vector [39] was replaced with the GALA sequence from pGaTB [40]. This pCaSpeR-GAL4 vector has unique restriction enzyme sites (kpnI, EcoRV, and BamHI) upstream of the GALA open reading frame. The 5.8-kb fragment, which includes the upstream promoter region and the first exon (to +557) of the dve gene, was inserted into the kpnI-BamHI sites of pCaSpeR-GAL4 to generate pCaSpeR-5.8EgA-GAL4. The 5.4-kb fragment, which includes further upstream sequences, was inserted into the kpnI-EcoRI sites of pCaSpeR-5.8EgA-GAL4 to generate pCaSpeR-5.4-5.8EgA-GAL4. The 1.6-kb EcoRI fragment was inserted into the junctional EcoRI site between 5.4-kb and 5.8-kb fragments of pCaSpeR-5.4-5.8EgA-GAL4 to generate the dve-13kb-GAL4. This construct expresses
GFP FRT40A/prd4 FRT40A (BZ-H1M, KEYENCE). Cross-section area was performed using a measurement module were obtained with an OLYMPUS FV300. Quantification of a TO-PRO3 (Invitrogen) was used to detect DNA. Confocal images were obtained with an OLYMPUS FV300. Quantification of a cross-section area was performed using a measurement module (BZ-H1M, KEYENCE).

Immunohistochemistry
Larvae, pupae, and adults were dissected in phosphate-buffered saline (PBS), fixed with 4% formaldehyde/PBS-0.3% Triton X-100 for 20 min, and washed several times with PBS-0.3% Triton X-100. The following primary antibodies were used: rabbit anti-Dve (1:1000) [24], rabbit anti-Ped (1:100, Asian Distribution Center for Segregation Antibodies) [41], mouse anti-Abd-B (1:10) (Developmental Studies Hybridoma Bank, DSHB), mouse anti-α-Spectrin (3A9) (1:50, DSHB), and mouse anti-β-galactosidase (1:200, Promega). FITC-, Cy3- or Cy5-conjugated secondary antibodies (Jackson Immunoresearch) were used for detection. TO-PRO3 (Invitrogen) was used to detect DNA. Confocal images were obtained with an OLYMPUS FV300. Quantification of a cross-section area was performed using a measurement module (BZ-H1M, KEYENCE).

Mosaic analyses
Mutant mosaic clones were induced with the use of FRT- and FLP-mediated recombination system [42] as following genotypes: w; FRT12D ubiGFP/FRT12D dve1; btl-GAL4/UAS-flp; y w hs-flp; ubi-GFP FRT40A/ped4 FRT40A with a heat shock pulse at 38°C-15 min. Mosaic clones expressing dve IR (y w hs-flp; Ay-GALA UAS-GFP.S65T/UAS-dve IR dve1) were induced with the Ay-GAL4 system [43] with a heat shock pulse at 38°C-20 min.

Behavior assays
All flies were cultured at 25°C under 12 hr light/dark cycles. Males and females were collected at eclosion and aged separately for 4–6 days in groups of 10–30 flies per vial. At circadian times 0 to 3 (CT0, subjective dawn), a pair of male and female flies was placed on a food medium with an observation chamber (30-mm diameter×4-mm depth) for 1 hr. A mated female was mildly aspirated into a vial and used for the following assays.

Progeny assay. Male fertility/fecundity was measured as the number of viable progeny per mated wild-type female during 4 days after mating. A mated female was transferred twice to a fresh vial and allowed to lay eggs for 2 days. The number of adult progeny from each vial was counted.

Egg-laying/hatching assay. A mated female was transferred to a fresh vial every 24 hr for 7 days, and eggs laid per day were counted. Hatching rate was determined by counting the number of unhatched eggs during next 24 hr.

Remating assay. At 4 hr and 48 hr after first mating, mated females were individually paired with one new virgin male (4–6 days old) for 1 hr. Several independent sets of remating assay were performed and the cumulative percentage of remated females was calculated.

Statistical analyses
The significance of differences between the control and test progenies was analyzed with t-tests or one-way ANOVA using Kaleidagraph software version 3.6 (HULINKS). The levels of significance are indicated by asterisks: *P<0.01, **P<0.001.

Supporting Information
Figure S1 RNAi-mediated dve knockout in the accessory gland. RNAi-mediated dve knockout (KD) greatly reduced Dve protein level (magenta) in the proximal (A) and the distal regions (B) at 72 hr APF. Cells inducing dve RNAi are marked by GFP expression (green: y w hs-flp; Ay-GALA UAS-GFP.S65T/UAS-dve IR dve1), and nuclei (DNA) are labeled with TO-PRO3 (blue).

Figure S2 Dve-dependent Abd-B expression during late pupal development. (A-D) Expression of Dve (A-D, magenta) and Abd-B (A′–D′) proteins in a male genital disc (A), accessory gland (AG) primordia at 24 hr APF (B), 48 hr APF (C), and AG of 1-day old adult (D). Abd-B is expressed in accessory gland precursors (A′) marked by btl expression (light blue in A′), and transiently repressed at 24 hr APF (B′). After 48 hr APF, expression patterns of Dve and Abd-B are nearly identical. MC: main cells (arrowheads), SC: secondary cells (arrows). (E) Abd-B expression (magenta in E′) is greatly reduced in dveIR null mutant clones at 72 hr APF. Mutant clones are marked by the absence of GFP expression (green) and are outlined in E″. Some wild-type cells also show reduced Abd-B expression in a cell-nonautonomous manner. ED: ejaculatory duct.

Acknowledgments
We are grateful to M. F. Wolfer, F. Karch, D. Gligrov, and J. Sitnik for sharing results prior to publication and for comments on the manuscript; T. Aigaki, the Bloomington Stock Center, the Drosophila Genetic Resource Center (DGRC, Kyoto), and the Vienna Drosophila RNAi Center (VDRC) for fly strains; N. Perrimon and C.S. Thummel for plasmid vectors; Y. Hiromi, the Asian Distribution Center for Segmentation Antibodies, and the Developmental Studies Hybridoma Bank (DSHB) for antibodies. We also thank the Advanced Science Research Center (Okayama University) for the use of confocal microscope OLYMPUS FV300 and fluorescence microscope KEYENCE BZ-9000.

Author Contributions
Conceived and designed the experiments: RM TAY HN. Performed the experiments: RM MW SS KT AK TI NW. Analyzed the data: RM TAY HN. Wrote the paper: RM HN.

References
1. Chapman T (2001) Seminal fluid-mediated fitness traits in Drosophila. Heredity 87: 511–521.
2. Gilott C (2003) Male accessory gland secretions: modulators of female reproductive physiology and behavior. Annu Rev Entomol 48: 163–184.
3. Wolfner MF (2002) The gifts that keep on giving: physiological functions and evolutionary dynamics of male seminal proteins in Drosophila. Heredity 88: 83–93.
4. Chapman T, Davies SJ (2004) Functions and analysis of the seminal fluid proteins of male Drosophila melanogaster fruit flies. Peptides 25: 1477–1490.
5. Bertram MJ, Akkarla GA, Ard RI, Gonzales C, Wolfer MF (1992) Cell type-specific gene expression in the Drosophila melanogaster male accessory gland. Mech Dev 38: 33–40.
6. Aigaki T, Fleischmann I, Chen PS, Kubli E (1991) Ecotopic expression of sex peptide alters reproductive behavior of female D. melanogaster. Neuron 7: 557–563.
7. Chapman T, Ranaghan J, Vinti G, Seifried B, Lung O, et al. (2003) The sex peptide of Drosophila melanogaster: female post-mating responses analyzed by using RNA interference. Proc Natl Acad Sci USA 100: 9925–9928.
8. Chen PS, Stumm-Zollinger E, Aigaki T, Balmer J, Bienz M, et al. (1988) A male accessory gland peptide that regulates reproductive behavior of female D. melanogaster. Cell 54: 291–298.
9. Kubli E (2003) Sex-peptides: seminal peptides of the Drosophila male. Cell Mol Life Sci 60: 1689–1704.
10. Liu H, Kahl E (2003) Sex-peptide is the molecular basis of the sperm effect in *Drosophila melanogaster*. Proc Natl Acad Sci USA 100: 9929–9933.

11. Hasemeyer M, Yapici N, Heberlein U, Dickson BJ (2009) Sensory neurons in the *Drosophila* genital tract regulate female reproductive behavior. Neuron 61: 511–518.

12. Yang CH, Rumpf S, Xiang Y, Gordon MD, Song W, et al. (2009) Control of the postmating behavioral switch in *Drosophila* females by internal sensory neurons. Neuron 61: 519–526.

13. Yapici N, Kim YJ, Ribeiro C, Dickson BJ (2008) A receptor that mediates the sperm bound sex-peptide controls female postmating behavior in *Drosophila*. Curr Biol 15: 207–213.

14. Peng J, Chen S, Buser S, Liu H, Honegger T, et al. (2005) Gradual release of sperm bound sex-peptide controls female postmating behavior in *Drosophila*. Nature 431: 13–37.

15. Ram KR, Wolfner MF (2009) A network of interactions among seminal proteins underlies the long-term postmating response in *Drosophila*. Proc Natl Acad Sci USA 106: 15384–15389.

16. Ahmad SM, Baker BS (2002) Sex-specific deployment of FGF signaling in *Drosophila* recruits mesodermal cells into the male genital imaginal disc. Cell 109: 631–641.

17. Xue L, Noll M (2002) Dual role of the Pax gene paired in accessory gland development of *Drosophila*. Development 129: 339–346.

18. Bertuccio C, Fasano L, Jun S, Wang S, Sheng G, et al. (1996) In vivo requirement for the paired domain and homeodomain of the paired segmentation gene product. Development 122: 2673–2683.

19. Snook RR, Hoeken EJ (2004) Sperm death and dumping in *Drosophila*. Nature 428: 939–941.

20. Xue L, Noll M (2000) *Drosophila* female sexual behavior induced by sterile males showing copulation complementation. Proc Natl Acad Sci U S A 97: 3272–3273.

21. Johnston RJ, Jr., Otake Y, Sood P, Behnia R, et al. (2011) Interlocked feedforward loops control cell-type-specific rhodopsin expression in the *Drosophila* eye. Cell 145: 956–968.

22. Nakagawa Y, Fujisawa-Fukuta S, Yorimitsu T, Tanaka S, Minami R, et al. (2011) Spatial and temporal requirement of Defective proventriculus activity during *Drosophila* midgut development. Mech Dev 128: 250–267.

23. Nakagoshi H (2005) Functional specification in the *Drosophila* endoderm. Dev Growth Differ 47: 383–392.

24. Nakagoshi H, Hoshi M, Nabeshima Y, Matsuzaki F (1998) A novel homeobox gene mediates the Dpp signal to establish functional specificity within target cells. Genes Dev 12: 2724–2734.

25. Nakagoshi H, Shirári T, Nabeshima Y, Matsuzaki F (2002) Refinement of wingless expression by a Wingless- and Notch-responsive homeodomain protein, Defective proventriculus. Dev Biol 249: 44–56.

26. Shirai T, Yorimitsu T, Kitirnooshi N, Matsuzaki F, Nakagoshi H (2007) Notch signaling relieves the joint-suppressive activity of Defective proventriculus in the *Drosophila* leg. Dev Biol 312: 147–156.

27. Yorimitsu T, Kitirnooshi N, Nakagoshi H (2011) Defective proventriculus specifies the ocellar region in the *Drosophila* head. Dev Biol 356: 598–607.

28. Ohkado T, Yamamoto MT (2011) Sperm of the *saetul* mutant are wasted when females utilize the stored sperm in *Drosophila melanogaster*. Genes Genet Syst 86: 97–108.

29. Bretman A, Lawieczak MK, Boone J, Chapman T (2010) A mating plug protein reduces early female remating in *Drosophila melanogaster*. J Insect Physiol 56: 107–113.

30. Villalta A, Peyre JB, Augaki T, Hall JC (2006) Defective transfer of seminal-fluid materials during matings of semi-fertile females mutants in *Drosophila*. J Comp Physiol A 192: 1253–1269.

31. Wigby S, Sirot LK, Linklater JR, Buchner N, Calboli FC, et al. (2009) Seminal fluid protein allocation and male reproductive success. Curr Biol 19: 751–757.

32. Taniguchi K, Kokuryo A, Imano T, Minami R, Nakagoshi H, et al. (2011) Binucleation of *Drosophila* adult male accessory gland cells increases plasticity of organ size for effective reproduction. J Organ Biol 1: e101.

33. Burglin TR, Casazza G (2002) Loss and gain of domains during evolution of cut superclass homeobox genes. Int J Dev Biol 46: 115–123.

34. Galande S, Dickinson LA, Mian IS, Sikorska M, Kohwi-Shigematsu T (2001) SATB1 cleavage by caspase 6 disrupts PDZ-domain-mediated dimerization, causing detachment from chromatin early in T-cell apoptosis. Mol Cell Biol 21: 5591–5604.

35. Gong F, Sun L, Wang Z, Shi J, Li W, et al. (2011) The BCL2 gene is regulated by a special AT-rich sequence binding protein 1-mediated long range chromosomal interaction between the promoter and the distal element located within the 3′-UTR. Nucleic Acids Res 39: 4640–4652.

36. Kuo TC, Chao CC (2010) Hepatitis B virus X protein prevents apoptosis of hepatocellular carcinoma cells by upregulating SATB1 and HURP expression. Biochem Pharmacol 80: 1093–1102.

37. Terricente J, Perea D, Suzanne M, Diaz-Benjumea FJ (2008) The *Drosophila* gene g92 is required to establish proximal-distal domains in the wing disc. Dev Biol 320: 102–112.

38. Jayaramaiah Raja S, Renkawitz-Pohl R (2005) Replacement by *Drosophila* melanogaster proteamines and Mst73F of histones during chromatin condensation in late spermatids and role of sesame in the removal of these proteins from the male pronucleus. Mol Cell Biol 25: 6165–6177.

39. Thummel CS, Boulet AM, Lipshitz HD (1988) Vectors for *Drosophila* P-element-mediated transformation and tissue culture transfection. Gene 74: 445–456.

40. Brand AH, Perrimon N (1993) Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development 118: 401–415.

41. Kosman D, Small S, Reinitz J (1996) Rapid preparation of a panel of polyclonal antibodies to *Drosophila* segmentation proteins. Dev Genes Evol 206: 183–185.

42. Xu T, Rubin GM (1993) Analysis of genetic mosaics in developing and adult *Drosophila* tissues. Development 117: 1223–1237.

43. Ito K, Awano W, Suzuki K, Hiromi Y, Yamamoto D (1997) The *Drosophila* mushroom body is a quadruple structure of clonal units each of which contains a virtually identical set of neurones and glial cells. Development 124: 761–771.