Supporting Information

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SI Materials and Methods

For immunohistochemical methods, fixed glands were incubated for 30 min in PBSTG blocking buffer (PBST containing 10% (vol/vol) filtered goat serum). Diluted primary antibody was incubated with the preparation overnight. The glands were washed 6× 10 min with PBST. Cy5-labeled secondary antibodies (Jackson Laboratories) were diluted 1:400 and incubated with the glands in the dark with gentle shaking for 2 h at 22 °C. TRITC-labeled phalloidin (Sigma; diluted 1:400 from a 1-mg/mL stock in methanol) was added to the secondary antibody solution when required. Glands were washed 5 × 10 min in PBST before being transferred to PBS for 10 min. The glands were mounted directly onto a microscope slide with a drop of DAPI-containing VectaShield (Vector Laboratories). Coverslips were placed over the glands and sealed in place with nail varnish. Female reproductive organs were also dissected in fix, but after washing, were then immediately mounted in DAPI-containing VectaShield.

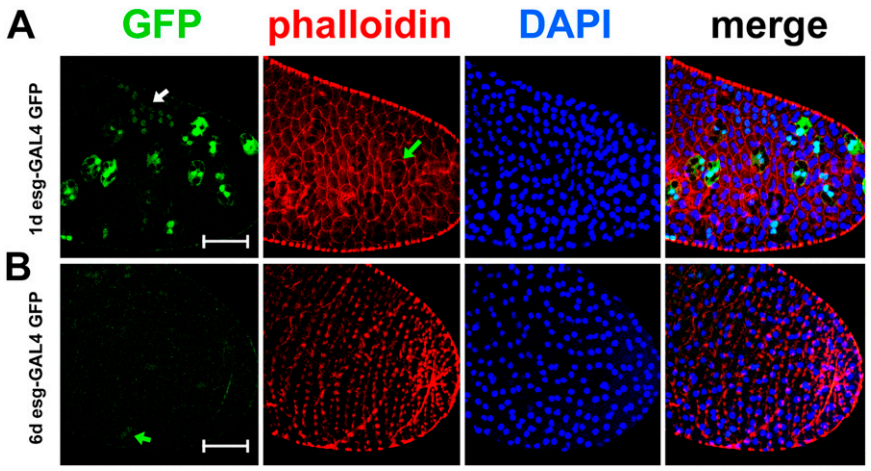

Fig. S1. The *esg-GAL4* driver is expressed transiently in adult secondary cells. Distal tips of the accessory gland from a 1-d-old (*A*) and 6-d-old (*B*) virgin adult male expressing nuclear GFP (green) under the control of *esg-GAL4*. Preparations were stained with TRITC-phalloidin (red), and DAPI (blue). GFP is primarily expressed in secondary cells (rounded cells; e.g., green arrow in *A*). It is also present at low levels in some main cells, but only for the first day of adulthood (white arrow). Only a few secondary cell nuclei contain detectable GFP at 6 d (green arrow in *B*). (Scale bars, 40 μm.) Images in these and all panels in Figs. S2, S4 *A–C*, S5*F*, S6, and S7 are representative of glands from at least 10 males of the same age and genotype.

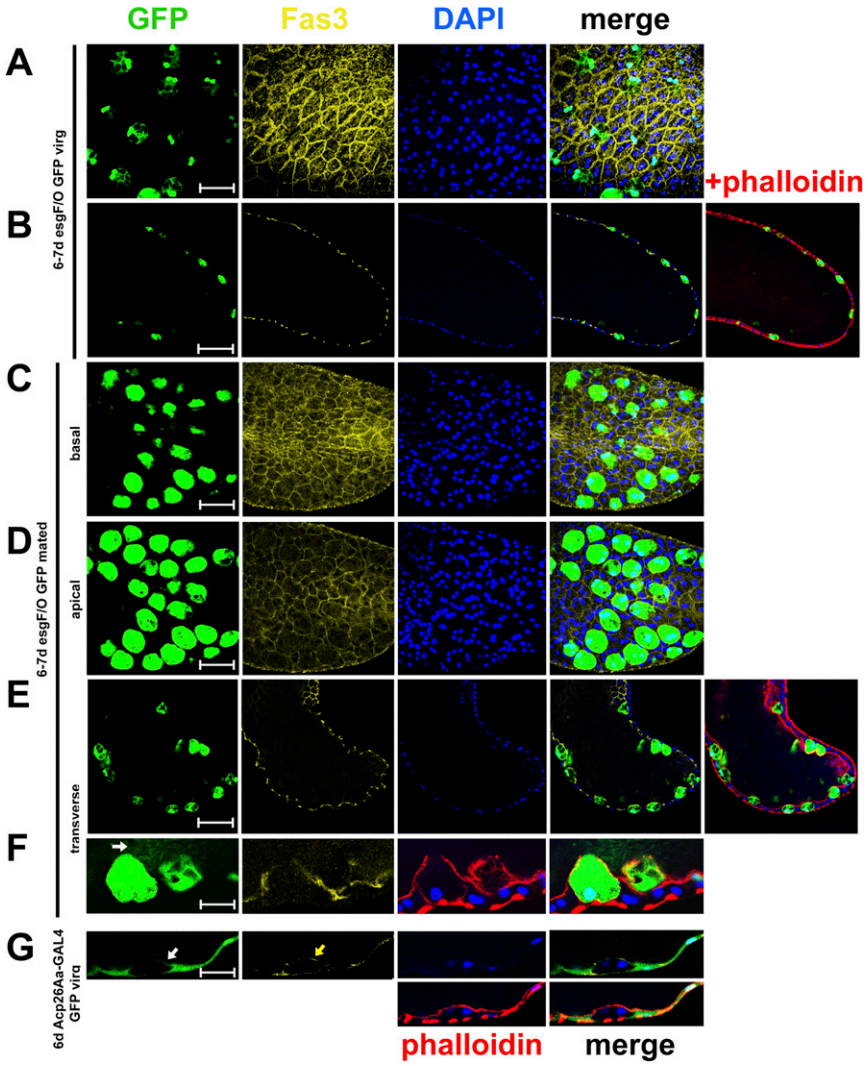

Fig. S2. Growth and delamination of secondary cells. (*A*–*F*) Distal tips of the accessory glands from 6- to 7-d-old virgin (*A* and *B*) or mated (*C*–*F*) males expressing nuclear GFP (green) under the control of *esg*^{ts} *Flp-Out* (*F/O*) after a temperature shift directly following eclosion. *B*, *E* and *F* are transverse confocal sections. Preparations were stained with TRITC-phalloidin (red), DAPI (blue), and an antibody to the septate junction protein Fas3 (yellow). Note the increased size of secondary cells in mated flies, seen most clearly in apical planes of section (*D*) and the release of cytoplasmic GFP into the lumen of the gland, observed in transverse section (arrow in *F*). Secondary cells round up along their apical-basal axis (*E* and *F*) and some detach from the underlying basal lamina (cell on right in *F*). (*G*) Transverse section of a secondary cell from the accessory gland of a 6-d-old virgin male expressing nuclear GFP in main cells under the control of *Acp26Aa-GAL4*; GFP is found in the nuclei and cytoplasm. Main cells extend their membranes apically to partially cover the surface of secondary cells (white arrow), reaching a position where the Fas3 septate junction protein is localized (yellow arrow). [Scale bars: 40 μm (*A*–*E*) and 20 μm (*F* and *G*).]

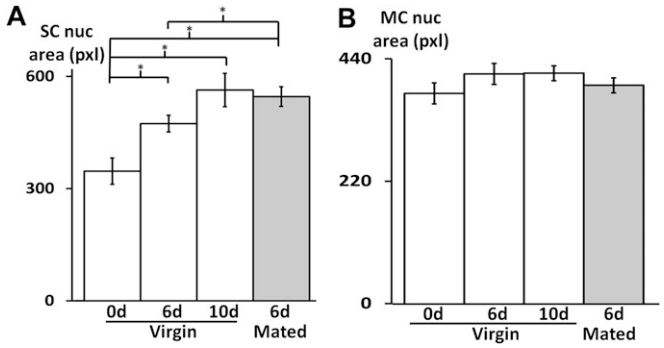

Fig. S3. Adult secondary cell, but not main cell, nuclei grow with age and after mating. Average secondary cell (*A*) and main cell (*B*) nuclear area (± 2× SEM) in images of accessory glands from newly eclosed, 6- and 10-d-old virgin males, and 6-d-old males that have been allowed to mate freely with at least six virgin females from eclosion. Change in relative nuclear size between secondary and main cells is plotted in Fig. 1*E*. *$P < 0.001$, $n = 5$ glands, one-way ANOVA.

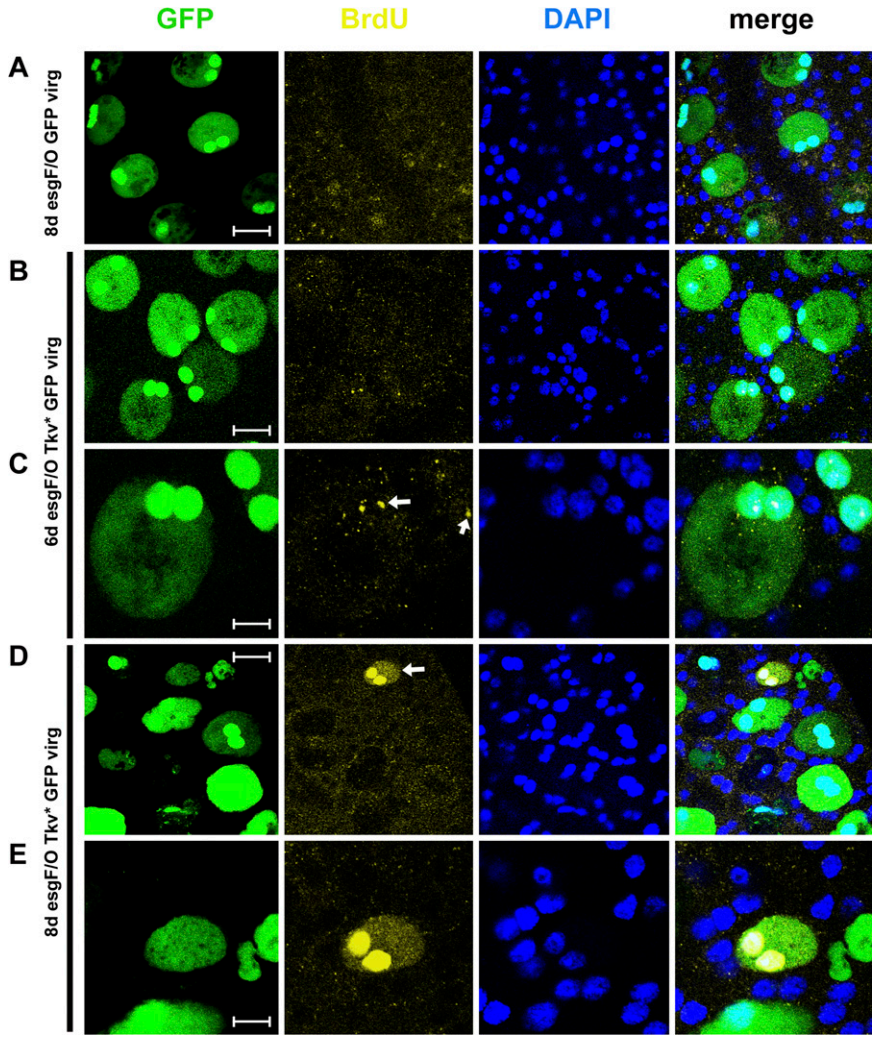

Fig. S4. Enlarged nuclear size in secondary cells from aged males and secondary cells with elevated BMP signaling is not associated with global DNA replication. Adult male flies expressing either GFP alone (*A*) or GFP and an activated form of bone morphogenetic protein (BMP) type I receptor Thick veins (Tkv) (*B*–*E*) under the control of esg^{ts} *F/O* after a temperature shift at eclosion were fed yeast paste containing BrdU. No incorporation of BrdU was detected in secondary cell nuclei with normal BMP signaling after 8 d (*A*), despite their significant growth (Fig. 1) and the fact that BrdU was incorporated into specific nuclei within the testis and midgut. Nuclei in most cells overexpressing activated Tkv also did not show BrdU incorporation (*D*), but occasionally they contained BrdU in two punctae that probably correspond to the nucleoli on the basis of DAPI staining (arrows in *C*), suggesting that some endo-reduplication may take place in these structures. (*D* and *E*) Example of a rare GFP^+ cell, which incorporates high levels of BrdU (arrow in *D*). Note that this cell does not have the morphology of a secondary cell, containing nuclei that are no larger than main cells, unlike all of the other GFP^+ secondary cells in this gland, and no large vacuoles: a single cell of this kind was seen in 2 of 20 glands expressing activated Tkv, but not in normal glands. DNA replication may be activated as a response to secondary cell loss in the former geneotype, but there is no evidence that these cells divide. The presence of a strongly labeled endo-reduplicating cell indicates that BrdU is accessible to the accessory gland, and that there is very little, if any, endo-reduplication taking place in the neighboring enlarged secondary cells. [Scale bars: 20 μm (*A*, *B*, and *D*), and 8 μm (*C* and *E*).]

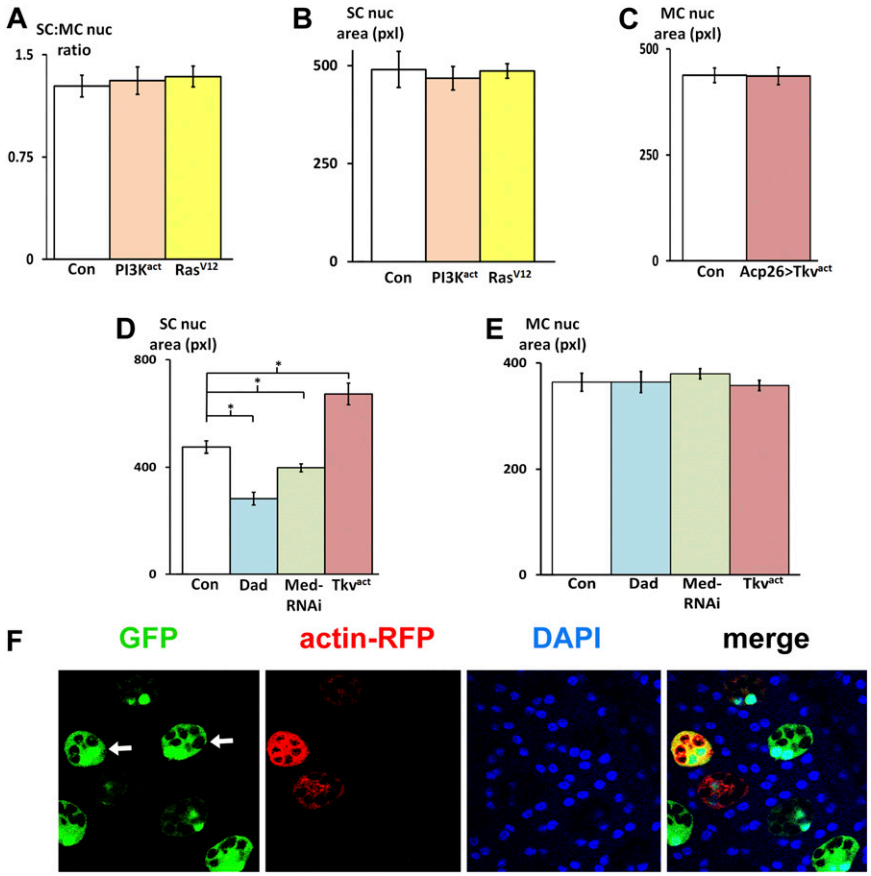

Fig. S5. BMP signaling promotes nuclear growth specifically in secondary cells. (*A* and *B*) The ratio of secondary cell nuclear area to main cell nuclear area and the absolute areas of secondary cell nuclei are unchanged when activated forms of the oncogenic proteins PI3K or Ras are expressed in adult secondary cells using the *esg*^{ts} *F/O* system. (*C*) Main cell nuclear area is unaffected by expressing an activated form of the BMP type I receptor Tkv in adults using the Acp26Aa-GAL4 driver under the control of GAL80^{ts}. (*D* and *E*) The absolute size of nuclei in secondary cells is affected by altered BMP signaling in these cells (*D*), but there is no effect of these treatments on neighboring main cell nuclei (*E*). (*F*) When the *esg*^{ts} *F/O* system is used in adults to drive a upstream activation sequences (UAS)-actin-RFP construct as well as UAS-GFP$_{nls}$, RFP levels clearly do not correspond to GFP levels in individual cells (compare two cells marked by arrows). Similar results are observed with other UAS-RFP constructs. Therefore, although GFP reliably marks secondary cells where the actin-F/O construct has been activated (e.g., Fig. S6*A*), the levels of expression do not indicate the expression levels of other UAS-regulated transgenes. Image width is 120 μm. For all experiments, males were temperature-shifted to activate GAL4 expression on the day of eclosion and areas were measured after 6 d of adulthood, except in *F*, where flies were dissected at 4 d. All data were found to be normally distributed and therefore analyzed by one-way ANOVA ($n = 5$), subsequently applying Bonferroni's correction for multiple comparisons. *$P < 0.001$ in *D*; $P > 0.1$ for all other comparisons with controls; error bars = ± 2× SEM.

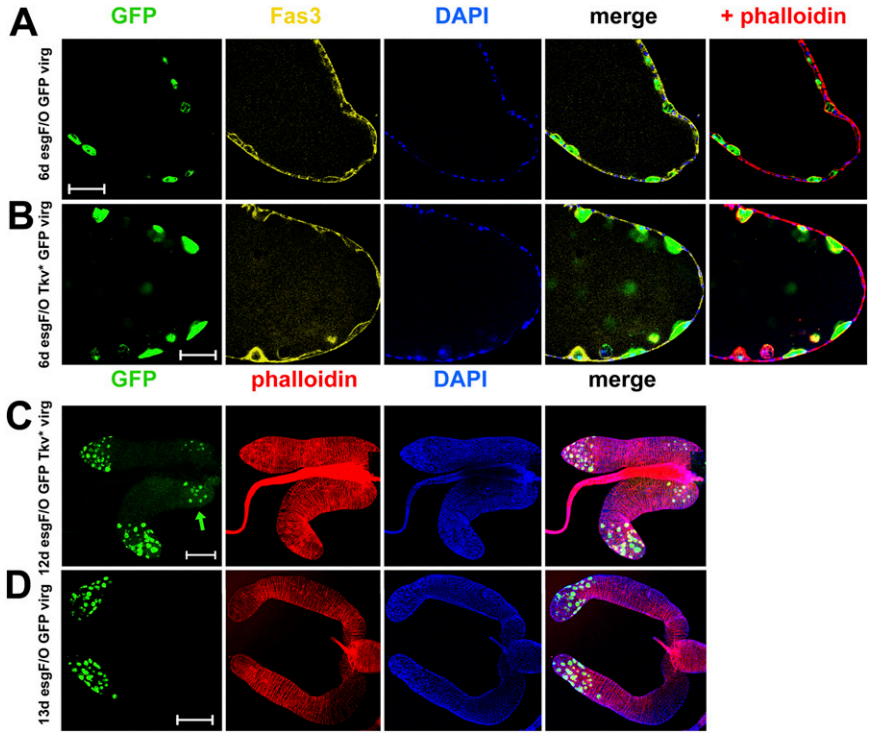

Fig. S6. BMP signaling promotes growth, delamination, and proximal migration of secondary cells. Images of accessory glands from 6-d-old (*A* and *B*; transverse sections) and 12- (*C*) or 13- (*D*) d-old virgin males expressing nuclear GFP with (*B* and *D*) or without (*A* and *C*) an activated form of the BMP type I receptor Tkv (Tkv*) under the control of esg^{ts} *F/O* after a temperature shift at eclosion. Preparations were stained with TRITC-phalloidin (red), DAPI (blue), and an antibody to the septate junction protein Fas3 (yellow). Secondary cells with increased BMP signaling are enlarged and have much larger nuclei than their neighbors (compare *B* with *A*). (*C* and *D*) A subset of adult secondary cells overexpressing activated Tkv under esg^{ts} *F/O* control in a 12-d-old (*L*) virgin male delaminates and migrates proximally (green arrow), a phenotype never observed in non-Tkv-expressing controls (*D*). [Scale bars: 40 μm (*A* and *B*) and 200 μm (*C* and *D*).]

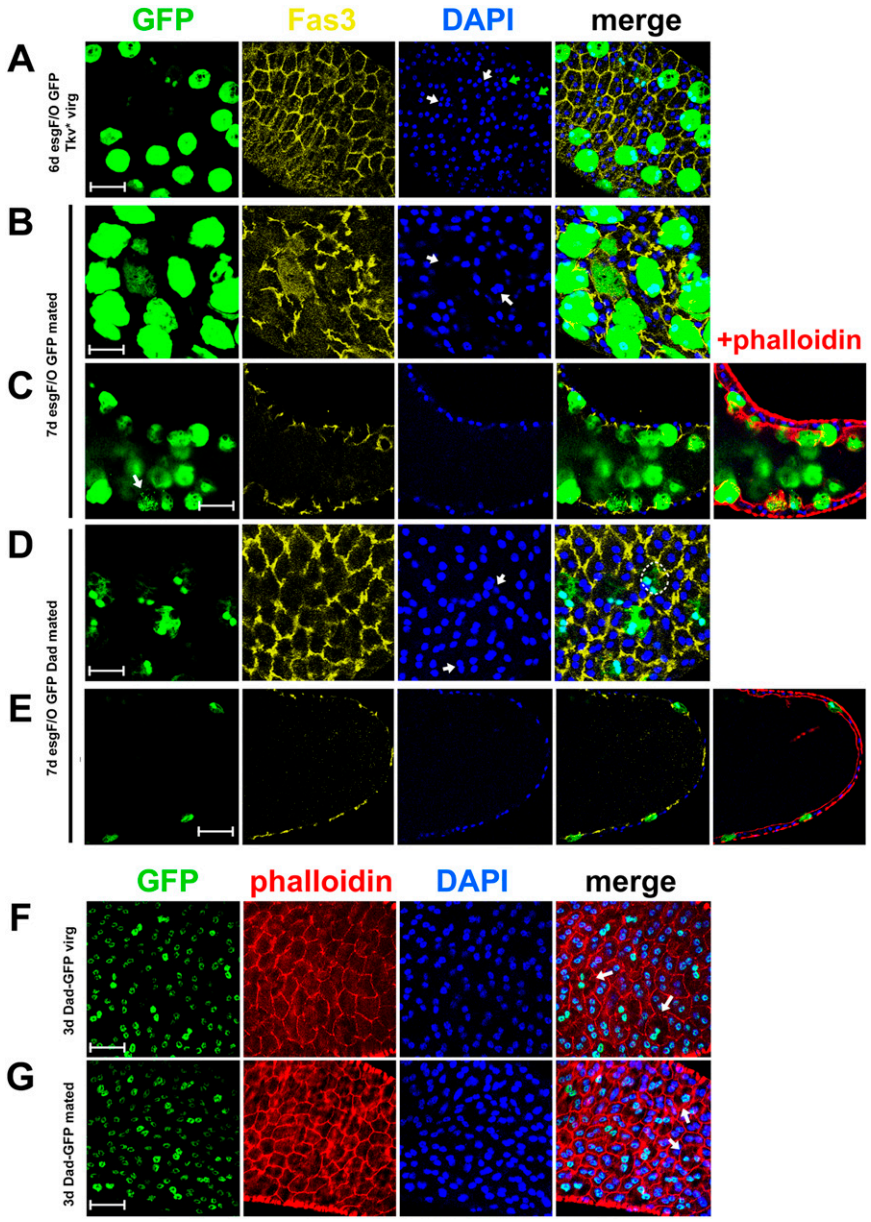

Fig. S7. BMP signaling in secondary cells is required for growth and delamination, but is not obviously up-regulated by mating. (*A*) Distal tip of an accessory gland from a 6-d-old virgin male expressing an activated form of Tkv and GFP under esg^{ts} *F/O* control. Flies were temperature-shifted from 18 °C to 28.5 °C at 3 d, so some secondary cells do not express high levels of GAL4 (e.g., white arrows; low levels of GFP are observed in some of these cells because of residual *esg-GAL4* activity in these males, which are roughly equivalent in age to adults that have been kept at 28.5 °C for 4.5 d). Nonexpressing cells are smaller and unlike the cells expressing activated Tkv (green arrows), their nuclei are not enlarged relative to their main cell neighbors. Preparations were stained with DAPI (blue) and an antibody to the septate junction protein Fas3 (yellow) or TRITC-phalloidin (red). (*B*–*E*) Images of accessory glands from 7-d-old multiply mated males expressing nuclear GFP with (*D* and *E*) or without (*B* and *C*) the BMP transcriptional repressor Dad under the control of esg^{ts} *F/O* after a temperature shift directly following eclosion. *C* and *E* are transverse confocal sections. Unlike normal secondary cells, cells expressing Dad do not grow relative to their neighbors, and their nuclei do not increase in size (arrows in *B* and *D*, respectively, with one of secondary cells outlined in merge in *D*). Furthermore, mating does not induce delamination of Dad-expressing secondary cells (*E*). (*F* and *G*) Images of accessory glands from 3-d-old males expressing nuclear GFP under the control of Dad regulatory elements, a reporter for BMP signaling. The male used for *G* was mated to a virgin female on day 3 and dissected 2 h afterward. Secondary cells (e.g., rounded cell outlines marked with arrows) normally express more GFP than main cells. In the mated male (*G*), there is no obvious change in BMP signaling activity compared with a virgin control of the same age (*F*), nor is there a change in males left for 4 h or overnight after a single mating or after multiple matings. [Scale bars: 40 μm (*A*, *C*, and *E*) and 20 μm (*B*, *D*, *F*, and *G*).]

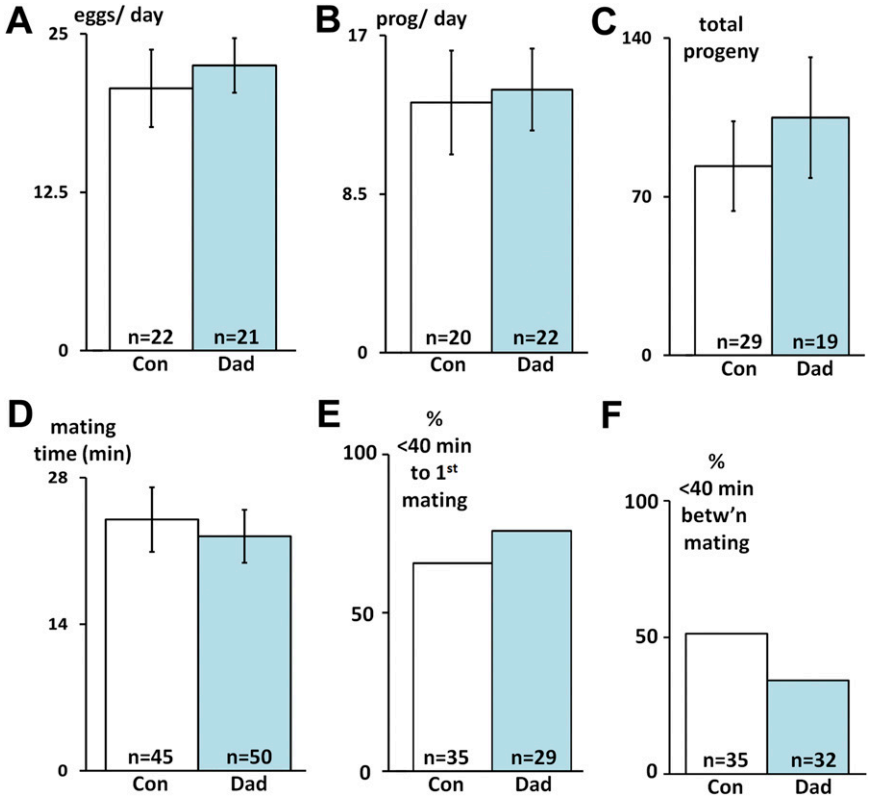

Fig. S8. Males expressing the BMP repressor Dad in adult secondary cells do not show altered mating behaviors and produce normal progeny numbers. (*A* and *B*) Charts show daily egg (*A*) and progeny (*B*) counts from females, each mated with a single 4- to 7-d-old male either expressing GFP in its secondary cells under the control of esg^{ts} *F/O* or expressing GFP and Dad. (*C*) Three-day-old males expressing either GFP or GFP and Dad in their secondary cells under esg^{ts} *F/O* control were mated individually to a group of five virgin females overnight. Each male was then separated and introduced to an additonal three virgin females for 24 h. Females from the first mating were individually separated into new vials to check that each female had mated. The females from the second round of mating were separated into individual vials and transferred every day for 1 wk. Chart shows the mean total number of progeny produced by these individual females. (*D*–*F*) Females were mated to multiply-mated males as in *C*, but mating was scored for 3 h after addition of the male to each group of three females. Charts show length of time for each mating observed (*D*), and the percentage of males that mate within 40 min of setting up the cross (*E*) and that remate within 40 min of a previous mating being initiated in the vial (*F*). Dad-expressing males show no significant differences in these assays. However, unlike controls, they do not prevent all females from remating 3 d later (Fig. 4*F*), even though the females that fail to be protected produce normal numbers of progeny in the 2 d following the first mating (Fig. 4*G*). For all experiments, males were temperature-shifted to activate GAL4 expression on the day of eclosion and then maintained at this temperature for matings. Data in *A*–*D* were subjected to a Mann–Whitney analysis. Data in *E* and *F* were analyzed by a two-tailed Fisher's exact test. Error bars = ± 2× SEM.