Supporting Information

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SI Materials and Methods

Fly Stocks and Genetics. *Oregon-R* was used as a wild-type strain. The following mutants and transgenic lines were used: *Notch*$^{264\text{-}39}$ (1) (a gift from S. Hayashi, RIKEN, Kobe, Japan), *Notch*^{5419} (a gift from K. Matsuno, Tokyo University of Science, Noda, Japan), *Ser*^{RX106} (2) (a gift from K. Matsuno), *Ser*^{CS94} (3) (a gift from K. Matsuno), *Dl*^{6B} (Bloomington Stock Center, Indiana University, Bloomington, IN), *Dl*^{B2} (Bloomington Stock Center), *Abd-B*^{M5} (4, 5) [*Drosophila* Genetic Resource Center, Kyoto, Japan (DGRC)], *neur*^{1}, *Egfr*^{tsla}, *Egfr*^{f24}, *spi*^{1} (Bloomington Stock Center), *stet*^{871} (6) (a gift from Y. Niki, Ibaraki University, Mito, Japan), *Df(3L)PX62* (6) (a gift from Y. Niki), *Star*^{JIN} (Bloomington Stock Center), *gcl*^{rev390} *cmp44E rescue#49* (7) (a gift from T.A. Jongens, University of Pennsylvania School of Medicine, Philadelphia, PA), *UAS-Notch*^{ICD} (8) (a gift from S. Hayashi), *UAS-Star* (Bloomington Stock Center), *UAS-Egfr*^{CA} (9) (a gift from L. Tsuda, National Institute for Longevity Sciences, Aichi, Japan), *UAS-2XEGFP* (Bloomington Stock Center), *hsp70-N-GV3* (10) (a gift from G. Struhl, Columbia University College of Physicians and Surgeons, New York, NY), *nanos-GAL4-VP16* (11) (a gift from M. Van Doren, Johns Hopkins University, Baltimore, MD), *twist24B-GAL4* (Bloomington Stock Center), and *twist-GAL4* (Bloomington Stock Center). These alleles were cultured at 25 °C. The genotypes of the mutant lines used in this study are as follows: *Notch*$^{264\text{-}39}$ *w*^{ch18A}*/FM7c P{Gal4-Kr.C} P{UAS-GFP.S65T} sn*, *Notch*^{5419} *FRT18A/FM7c P{Gal4-Kr.C} P{UAS-GFP.S65T} sn*, *Ser*^{RX106}*/TM3 P{Gal4-Kr.C} P{UAS-GFP.S65T}*, *ri Ser*^{CS94}*/TM3 P{Gal4-Kr.C} P{UAS-GFP.S65T}*, *Dl*^{B2} *e*^{1}*/TM3 P{Gal4-Kr.C} P{UAS-GFP.S65T}* (*Dl*^{B2}; an amorphic mutation), *ss*^{1} *Dl*^{6B} *e*^{1}*/TM3 P{Gal4-Kr.C} P{UAS-GFP.S65T}* (*Dl*^{6B}; a hypomorphic mutation), *mwh*^{1} *jv*^{1} *st*^{1} *red*^{1} *Sb*$^{sbd\text{-}2}$ *e*^{11} *ro*^{1} *ca*^{1} *Abd-B*^{M5}*/TM3 P{Gal4-Kr.C} P{UAS-GFP.S65T}* (*Abd-B*^{M5}; a hypomorphic mutation), *Egfr*^{tsla}*/CyO P{Gal4-Kr.C} P{UAS-GFP.S65T}*, *Egfr*^{f24}*/CyO P{Gal4-Kr.C} P{UAS-GFP.S65T}* (*Egfr*^{f24}; an amorphic mutation), *Star*^{JIN} *cn*^{1} *bw*^{1} *sp*^{1}*/CyO P{Gal4-Kr.C} P{UAS-GFP.S65T}* (*Star*^{JIN}; an amorphic mutation), *spi*^{1} *cn*^{1} *bw*^{1} *sp*^{1}*/CyO P{Gal4-Kr.C} P{UAS-GFP.S65T}* (*spi*^{1}; a hypomorphic mutation), *neur*^{1}*/TM3 P{Gal4-Kr.C} P{UAS-GFP.S65T}* (*neur*^{1}; a hypomorphic mutation), *stet*^{871}*/TM3 P{Gal4-Kr.C} P{UAS-GFP.S65T}* (*stet*^{871}; a hypomorphic mutation), and *Df(3L)PX62/TM3 P{Gal4-Kr.C} P{UAS-GFP.S65T}*. The genotypes of embryos were determined by using the GFP-expressing balancer chromosomes.

Egfr^{ts} is an allelic combination of *Egfr*^{tsla} and *Egfr*^{f24}. *Egfr*^{tsla}*/CyO P{Gal4-Kr.C} P{UAS-GFP.S65T}* virgins were crossed with *Egfr*^{f24}*/CyO P{Gal4-Kr.C} P{UAS-GFP.S65T}* males at 18 °C, and the embryos at 14–20 h after egg laying (AEL) were collected. They were then incubated at 29 °C in a humidified chamber for 7 h.

To detect Notch activation, we used *yw hs-flp; hsp70-N-GV3/UAS-2XEGFP* flies (10). The embryos were heat-shocked at 6.5–9.5 h AEL at 37 °C twice for 30 min, with an intervening 1 h interval at 25 °C, and were allowed to develop to stage 14–17 at 25 °C.

Staging of embryos was conducted as previously described (12).

In Situ Hybridization. In situ hybridization was performed as previously described (13). DIG-labeled antisense RNA probes were synthesized from PCR products amplified from pGEM-T Easy Vector (Promega) containing cDNA fragments with T7 and SP6 primers. The cDNA fragments were amplified from an embryonic cDNA library (14) by using the following primers:

Ser: 5′-TTTAGTCGAGCGCCGTGCTTCGAGCGG-3′ and 5′-CTAAACCATCACAGTGGTGGCAAGGAC-3′;
neur: 5′-CCCTCTTCATGTCCTGGCCCCAACAAC-3′ and 5′-CCATTTTCCATATTTCATTCAAGCTGTTGG-3′;
kek1: 5′-GCCAGTGTGTGCAATGGCAATGGGC-3′ and 5′-GACCGTGAACTGCCGCCCCCGCCACTG-3′;
spi: 5′-CTCAACGTTTACGTTCCACCAGCAGC-3′ and 5′-GGCGCGTGTGTCGCGTTGTGTGTGTG-3′;
stet: 5′-GGCCGGACAAGTGCATCCGTCGGCAGT-3′ and 5′-GATGCTAGTGCCCAGGCTTCCCGCC-3′; and
Star: 5′-CGCGCGACTACGAGCTGAATGGGGTTGCGC-3′ and 5′-ATGTGCATGAGTGTAGTGTGAGTG-3′.

Triple staining for *kek1* RNA, the germ-line marker Vasa (Vas), and GFP was performed as previously described (15).

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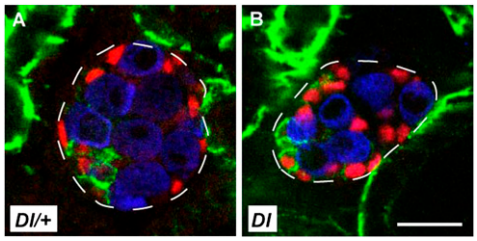

Fig. S1. Ectopic hub differentiation was undetectable in the male embryonic gonads of *Dl* mutant embryos. Male gonads in Dl^{B2}/+ (*A*) and Dl^{B2}/Dl^{6B} (*B*) embryos (stage 16/17) stained for Fas3 (green), Tj (red; a marker for SGPs), and Vas (blue). (Scale bar, 10 μm.) The average number of Fas3-positive cells ± SD per gonad at stage 16/17 was 7.9 ± 2.3 in Dl^{B2}/+ embryos and 7.2 ± 2.6 in Dl^{B2}/Dl^{6B} embryos (20 gonads were examined in each case). These values were not significantly different ($P > 0.05$, Student's *t* test).

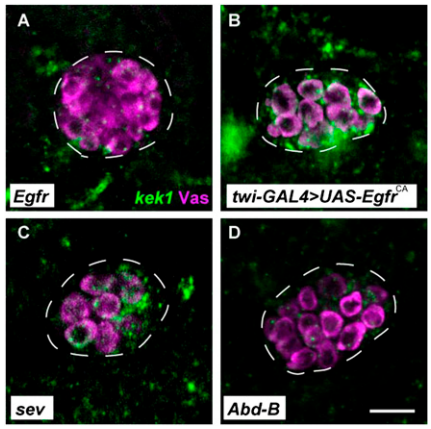

Fig. S2. *kek1* RNA expression in male embryonic gonads. Male gonads in $Egfr^{tsla}/Egfr^{f24}$ (*A*), *twist-GAL4* > $UAS\text{-}Egfr^{CA}$ (*B*), sev^{d2}/Y (C), and $Abd\text{-}B^{M5}/Abd\text{-}B^{M5}$ (*D*) embryos (stage 16) stained for *kek1* RNA (green) and Vas (magenta). Anterior is to the left, and gonads are outlined by white lines. (Scale bar, 10 μm.)