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Sightless has homology to transmembrane acyltransferases and is required to generate active Hedgehog protein

Jeffrey D. Lee and Jessica E. Treisman

Proteins of the Hedgehog (Hh) family act as important developmental signals in a variety of species [1]. Hh proteins are synthesized as full-length precursors that are autocatalytically cleaved by their C-terminal domains to release the signaling N-terminal domains [2]. The addition of a cholesterol molecule to the C terminus of the signaling domain is concomitant with cleavage [3]. Vertebrate Sonic hedgehog (Shh) proteins have also been shown to acquire a fatty acid chain on the N-terminal cysteine of this domain [4], which is required for a subset of their in vivo functions [5, 6]. A mutation of the corresponding cysteine in *Drosophila* Hh transforms it into a dominant-negative protein [6]. We have identified a novel gene, *sightless* (*sit*), which is required for the activity of *Drosophila* Hh in the eye and wing imaginal discs and in embryonic segmentation. *sit* acts in the cells that produce Hh, but does not affect *hh* transcription, Hh cleavage, or the accumulation of Hh protein. *sit* encodes a conserved transmembrane protein with homology to a family of membrane-bound acyltransferases. The Sit protein could act by acylating Hh or by promoting other modifications or trafficking events necessary for its function.

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Results and discussion

sightless is required for the activation of Hedgehog target genes

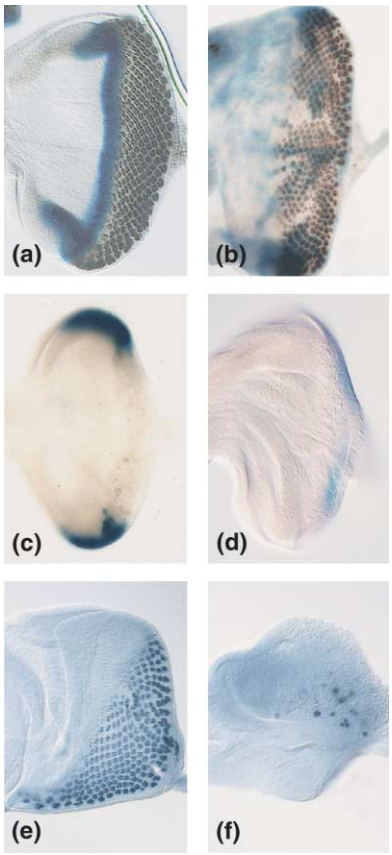
In a mosaic screen for novel genes required for *Drosophila* photoreceptor differentiation [7–9], we identified two alleles of a gene that we named *sightless* (*sit*) for its effect on photoreceptor development. Clones of *sit* mutant cells in the eye disc showed a reduction in the number of Elav-expressing photoreceptors that was most pronounced near the center of large clones (Figure 1b), suggesting that *sit* might act nonautonomously. Because both *sit* alleles caused pupal lethality, we were able to examine the eye discs of third instar larvae transheterozygous for two *sit* alleles. In these discs, only a few cells were able to differentiate as photoreceptors (Figure 1c). Rescue by adjacent wild-type tissue may thus contribute to the differentiation observed in *sit* mutant clones.

One of the critical signals triggering photoreceptor development is Hedgehog (Hh), which is expressed at the posterior margin of the disc prior to differentiation and subsequently in the differentiating photoreceptors [10–14]. Hh activates the expression of *decapentaplegic* (*dpp*) in a stripe at the front of differentiation, or morphogenetic furrow; Dpp signaling also promotes photoreceptor formation [10, 12, 15–19]. *dpp* expression was lost from the morphogenetic furrow in *sit* mutant eye discs (Figure 1c). Another target of Hh signaling, the proneural gene *atonal* [20, 21], also required *sit* for its expression (data not shown). Despite this lack of Hh target gene expression, a *hh-lacZ* enhancer trap [22] was expressed at the posterior margin of *sit* mutant eye discs (Figure 1d), indicating that *hh* expression is established normally. This suggests that the *sit* phenotype could be due to a defect in Hh signaling.

Hh signaling has been extensively studied in the wing disc, where *hh* is expressed in the posterior compartment and signals to cells just anterior to the compartment boundary to upregulate the expression of *dpp* and *patched* (*ptc*) (Figure 2a,c,g) [23, 24]. The Hh signal is mediated by the stabilization and activation of the full-length form of the transcription factor Cubitus interruptus (Ci) [25]. This stabilization can be detected with an antibody directed against the C-terminal region of Ci, which fails to recognize the cleaved form of Ci produced in the absence of Hh signaling (Figure 2e) [25, 26]. *sit* mutant wing discs showed defects consistent with a lack of Hh pathway function; *ptc* expression was not upregulated at the compartment boundary, and *dpp* expression was almost completely absent (Figure 2b,d). In addition, we failed to detect any stabilization of full-length Ci at the compartment boundary (Figure 2f). However, we found that *hh-lacZ* was expressed at wild-type levels in *sit* mutant discs, indicating that *hh* transcription was unaffected (Figure 2g,h). This implicates Sit in the Hh pathway downstream of *hh* transcription and upstream of Ci stabilization.

The defects in *sit* mutant discs appeared to be specific to anterior-posterior patterning; *wingless* (*wg*), which marks the dorsal-ventral compartment boundary, and its target gene *Distal-less* [27, 28] were still expressed in *sit* mutant wing discs (data not shown). In addition, the phenotype was not as severe as the complete loss of *hh* from early

Figure 1



sightless is required for photoreceptor differentiation. All panels show third larval instar eye imaginal discs with the posterior oriented toward the right. **(a)** Wild-type, **(b)** sit^{T802} mosaic clones, and **(c,d)** sit^{T392}/sit^{T802}. Panels (a)–(c) are stained with anti-Elav antibody in brown to reveal differentiating photoreceptors, which are reduced in number in clones homozygous for *sit* and almost completely lacking in larvae homozygous for *sit*. Blue X-gal staining reveals *dpp-lacZ* expression in (a) and (c) and *arm-lacZ* expression marking wild-type tissue in (b). *dpp* expression is lost from the morphogenetic furrow in *sit* mutant discs. Panel (d) is stained with X-gal to reveal *hh-lacZ* expression at the posterior margin of the *sit* mutant eye disc. **(e)** *ey*-GAL4/+; UAS-*Hh-N*/+, and **(f)** *ey*-GAL4/+; UAS-*Hh-N*, sit^{T392}/sit^{T392}. Panels (e)–(f) are stained with anti-Elav antibody. The expression of Hh-N in a wild-type background dorsalizes the eye disc and induces premature photoreceptor differentiation, but it has no significant effect in a *sit* mutant background.

stages of larval development; although *sit* discs were smaller than wild-type discs, they were larger and more normally shaped than *hh* mutant discs [23, 29]. Our *sit* alleles are therefore likely to cause an incomplete or late loss of Hh signaling; since they appear to be nulls at the molecular level (see below), this may be due to the activity of maternally contributed *sit*.

Hh signaling is also required for normal embryonic segmentation; *hh* mutant embryos show a loss of naked cuticle and of *wg* expression [30, 31]. When we removed the maternal contribution of *sit* by making germline clones, we observed a loss of naked cuticle strongly resembling the *hh* phenotype; however, a wild-type copy of *sit* provided on the paternal chromosome was able to fully rescue the phenotype (data not shown). In embryos lacking both maternal and paternal *sit*, stripes of Wg expression were lost from the ectoderm by stage 11 (Figure 2j). Thus, *sit* is required for the expression of Hh target genes in the embryo as well as in the eye and wing discs.

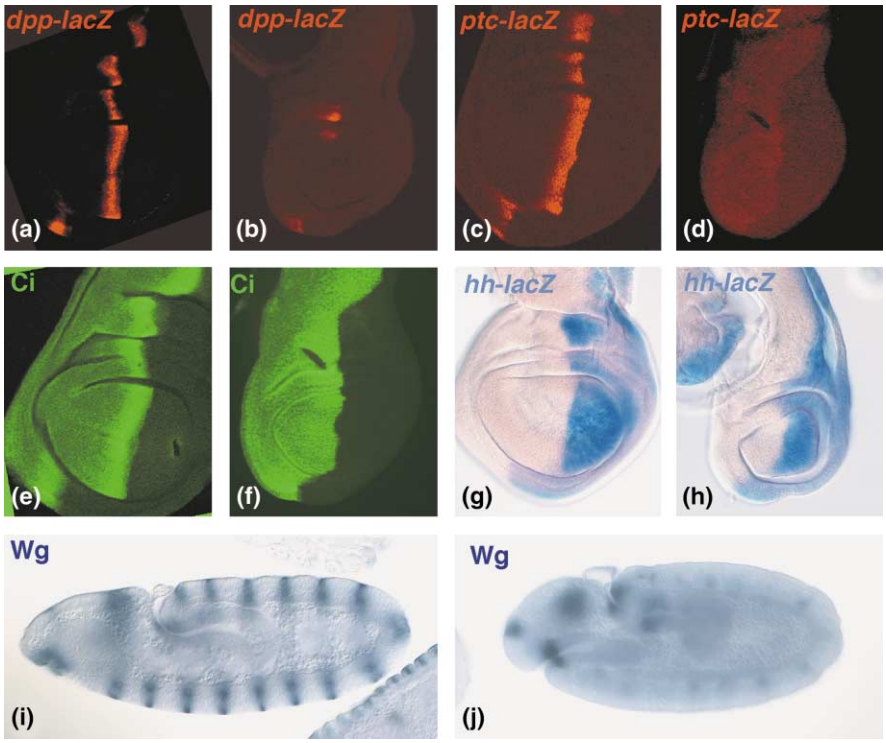
sit is required in the Hh-producing cells but does not affect the level of Hh protein

sit might affect Hh signaling by promoting the production of functional Hh or by allowing cells to respond to the Hh signal. To distinguish between these possibilities, we used mosaic analysis to determine in which cells *sit* function was required; in the wing disc, Hh-producing cells are restricted to the posterior compartment, and Hh-responding cells are restricted to the anterior compartment. We found that small clones of cells homozygous for *sit* had no effect on *ptc* or *dpp* expression in the wing disc (data not shown), consistent with the nonautonomy of *sit* function in the eye disc. When we used the *Minute* technique [32] to generate larger clones lacking *sit*, we found that *sit* function was not required in the *ptc*-expressing cells or anywhere in the anterior compartment for *ptc* upregulation, provided that *sit* was present in the posterior compartment (Figure 3a–c). We also found that the loss of *sit* from the posterior compartment prevented *ptc* upregulation in adjacent anterior cells even if they themselves were wild-type for *sit* (Figure 3d–f). Thus, *sit* function in cells of the posterior compartment is both necessary and sufficient to upregulate *ptc* in anterior compartment cells. This suggests that *sit* may be required for the production, activity, or release of Hh protein.

To determine whether Hh protein could be produced in the absence of *sit* function, we stained *sit* mutant clones in the wing disc with an antibody to the N-terminal domain of Hh. No change in the intensity of staining was apparent in *sit* mutant clones compared to adjacent wild-type tissue (Figure 3g–i). Thus, *sit* is not required for Hh translation or stability. In clones that are mutant for *dispatched* (*disp*), which encodes a protein required for Hh release from the cell, Hh protein accumulates to high levels [33]. We did not observe such an accumulation of Hh in *sit* mutant clones, suggesting that unlike Disp, Sit does not act at the level of Hh release.

Hh is synthesized as a full-length precursor that is then cleaved by the autocatalytic activity of its C-terminal domain to release the N-terminal signaling domain [2, 34]. *sit* does not appear to be required for this cleavage, as similar proportions of full-length Hh and its cleaved N-terminal domain were detected on Western blots of extracts from *sit* mutant and wild-type third instar larvae (data not shown). We also tested whether the expression

Figure 2



sit is required to activate Hh target genes. **(a–h)** Third instar wing discs with the posterior oriented toward the right and the dorsal side facing up. (a,c,e,g) Wild-type, and (b,d,f,h) sit^{T392}/sit^{T802}. (a,b) Anti-β-galactosidase staining shows *dpp-lacZ* expression, and (c,d) anti-β-galactosidase staining shows *ptc-lacZ* expression. *dpp* expression is almost absent, and *ptc* is not upregulated at the compartment boundary in *sit* mutant discs. (e,f) Anti-Ci staining. Upregulation of full-length Ci at the compartment boundary does not occur in *sit* mutant discs. (g,h) X-gal staining shows *hh-lacZ* expression, which is unaffected by the absence of *sit*. Panels **(i)–(j)** show stage 11 embryos stained with anti-Wg antibody. (i) Wild-type, and (j) a sit^{T392} germline clone without paternal rescue. The Wg stripes fade early, as they do in *hh* mutant embryos.

of an N-terminally truncated form of Hh (Hh-N) could rescue *sit* mutants; this form of the protein is not cholesterol-modified or restricted in its diffusion [35] and does not require *disp* for its release from the cell [33]. The expression of UAS-*Hh-N* with *eyeless*-GAL4 could induce premature photoreceptor differentiation in wild-type eye discs (Figure 1e) but did not alter the phenotype of *sit* mutant eye discs (Figure 1f). These results suggest that *sit* is required for Hh activity, but not for its cleavage, cholesterol modification, or secretion.

sit encodes a conserved transmembrane protein with homology to acyltransferases

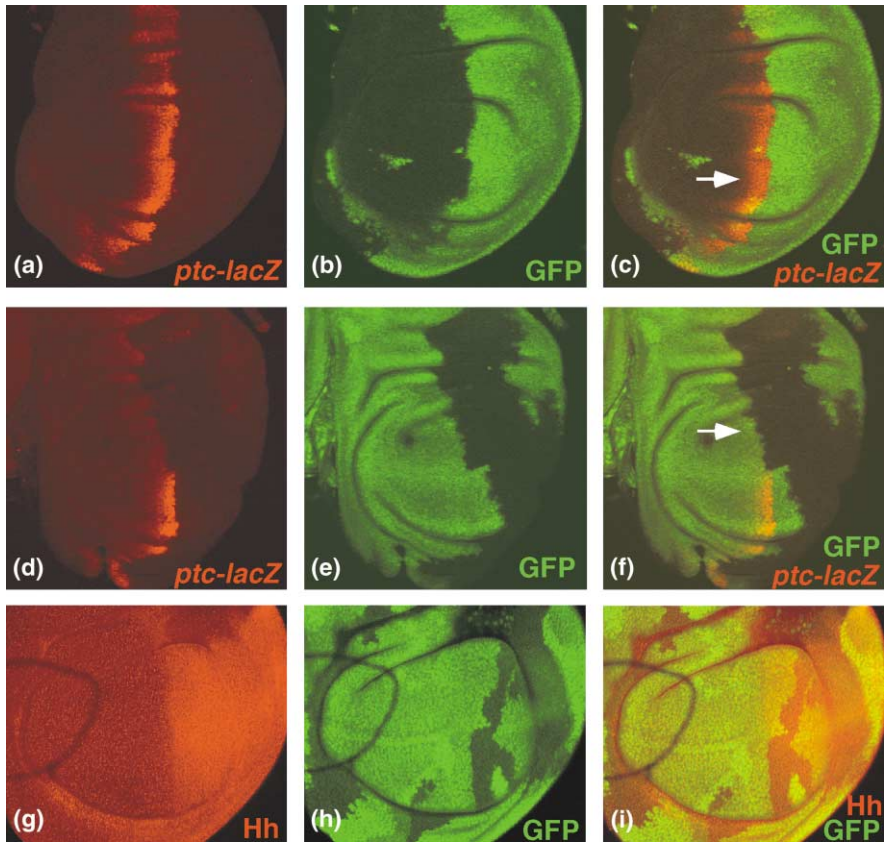
To identify the *sit* gene, we first mapped it by complementation tests with a collection of deficiencies. We found that the deficiency *Df(3L)M21* failed to complement the lethality of *sit* alleles, localizing *sit* to the 62F–63B region. We then used P element-mediated male recombination mapping [36] to further refine this map position (Figure 4a). We found that *sit* mapped to 63B11–13, between the P elements *l(3)S103012* and *l(3)j5c2*. Eleven predicted genes lie in this region. We found that each of our two *sit* alleles had a sequence change in its genomic DNA that would introduce a stop codon into the predicted protein encoded by one of these genes, *CG11495* (Figure 4a,b). These changes were not present in the isogenic strain used for the original mutagenesis. The *sit* mutant phenotype is thus likely to be caused by loss of function of CG11495.

Since no ESTs corresponding to CG11495 had been listed in the Berkeley *Drosophila* genome project database, we screened an embryonic cDNA library with a PCR probe from the 5′ end of the predicted open reading frame. We isolated three cDNAs containing the full open reading frame (Figure 4b). In situ hybridization showed that this transcript was expressed uniformly at low levels in the imaginal discs and early embryo (data not shown). The encoded protein has ten predicted transmembrane domains and shows homology to human, mouse, and *C. elegans* proteins present in the database. Its closest human homolog is BAA91772, to which it shows 28% identity and 45% similarity (Figure 4b). In addition, the Sit protein shows more distant homology to a family of proteins that have been shown to transfer acyl chains onto hydroxyl groups of membrane-bound lipid or protein targets [37] (Figure 4c). An invariant histidine that was suggested as a possible active-site residue [37] is conserved in the Sit sequence, and both *sit* mutations would truncate the protein prior to the region of acyltransferase homology.

Possible functions of Sit

We have shown that *sit* encodes a putative transmembrane protein required in Hh-producing cells to generate active Hh protein. Since *sit* does not alter the level of Hh protein present in the cell, it is unlikely to affect Hh translation or release. The cleavage of Hh to release the N-terminal signaling domain also does not require *sit*, and an exoge-

Figure 3



sit is required in the Hh-producing cells but does not alter the level of Hh protein. All panels show third instar wing discs containing sit^{T392} mutant clones oriented as in Figure 2. Wild-type tissue is marked by GFP expression in green in **(b,c,e,f,h,i)**. *ptc-lacZ* expression is monitored by red anti-β-galactosidase staining in **(a,c,d,f)**. The arrow in (c) points to *sit* mutant cells that are able to express *ptc*. The arrow in (f) points to wild-type cells just anterior to the compartment boundary that do not express *ptc*. Panels **(g)** and (i) show anti-Hh staining in red. No change in the amount of Hh protein is apparent in *sit* mutant cells.

nously provided Hh-N domain is inactive in the absence of *sit*. *sit* is unlikely to be required for cholesterol addition to the C terminus of the signaling domain; bacterially produced Hh protein becomes cholesterol-modified in vitro, and this modification restricts Hh localization, but does not increase its activity in vivo [2, 3, 35].

Human and rodent Sonic hedgehog (Shh) proteins have been shown to acquire a palmitoyl modification on the N-terminal cysteine of the signaling domain in cell culture [4]. Mutation of this cysteine to serine in human Shh prevents its palmitoylation and greatly reduces its ability to ventralize the mouse forebrain [4, 5]. We have previously shown that the corresponding cysteine to serine mutation in *Drosophila* Hh (Hh-C84S) completely abolishes its activity; however, the mutant protein appears to be secreted and appears to block the effects of wild-type Hh in the extracellular space [6]. Together with the homology of Sit to acyltransferases, this raises the intriguing possibility that Sit might be the enzyme responsible for the palmitoylation of Hh. However, this would represent a difference in specificity between Sit and the other acyltransferases of this family, which acylate hydroxyl groups; the inactivity of Hh-C84S indicates that a hydroxyl group cannot act as a substrate in this case. Further biochemical analysis will be needed to determine whether *Drosophila* Hh is in fact palmitoylated and whether this palmitoylation requires *sit* function.

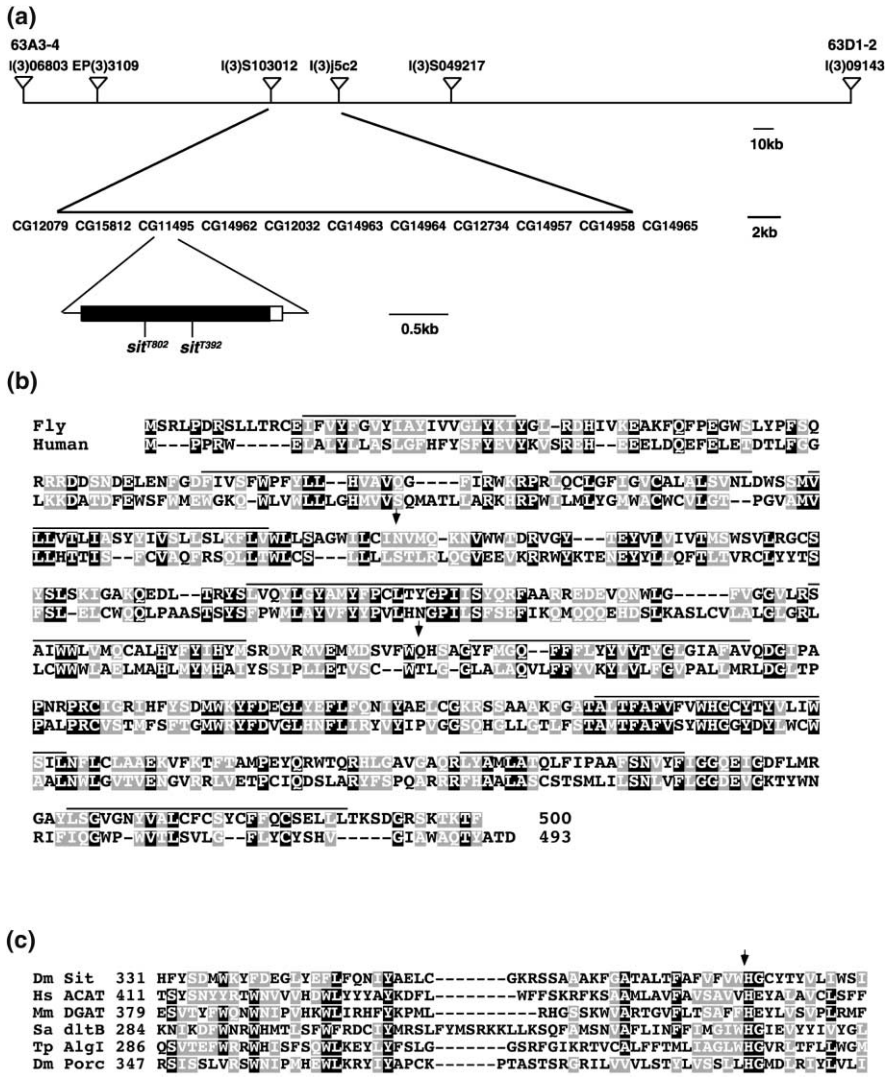
Alternatively, Sit could be required for another modification or trafficking event required for Hh activity. Sit is distantly related to the Porcupine (Porc) protein, which is required in Wg-producing cells for Wg activity. Porc is localized to the endoplasmic reticulum and alters the glycosylation state of Wg [38, 39]. Interestingly, Porc also has homology to the membrane-bound O-acyltransferase family [37] (Figure 4c). The requirement of Porc for Wg function and Sit for Hh function suggests that modifications that are essential for the activity of signaling proteins may be more widespread than previously believed.

Materials and methods

Genetics

The fly strains that were used included *hh-lacZ* [22], *dpp-lacZ* [40], *ptc-lacZ* [41], *ey*-GAL4 [42], and UAS-*Hh-N* [35]. To make *sit* clones in the eye disc, FRT80B, *sit*/TM6B males were crossed to FRT80B, P(*arm-lacZ*); *ey*FLP1 females. To make large *sit* clones in the wing disc, FRT80B, *sit*/TM6B males were crossed to FRT80B, *M(3)67C*, *Ubi*-GFP/TM6B; *hs*FLP122 females, and larvae were heat shocked for 1 hr at 38.5°C in the first and second instar. To make *sit* germline clones, an insertion of P(ovo^D) was transposed onto the FRT80B chromosome, and FRT80B, *sit*/TM6B females were crossed to FRT80B, P(ovo^D)/TM3; *hs*FLP122/Y

Figure 4



Sit is a conserved transmembrane protein with homology to acyltransferases. **(a)** The identification of the *sit* gene. *sit* was mapped by P element-induced male recombination with each of the P elements shown, localizing it to a region containing 11 predicted genes between *l(3)S103012* and *l(3)j5c2*. The sequence of the CG11495 coding region was altered in DNA from each of the two *sit* alleles. **(b)** The alignment of the Sit protein sequence (fly) with its closest human homolog, BAA91772 (human). Identical amino acids appear white on a black background, while conserved residues appear white on a gray background. Predicted transmembrane domains are overlined. Arrows mark the positions of the sequence changes in sit^{T802} (N156) and sit^{T392} (Q287). Both changes would introduce stop codons into the predicted protein. **(c)** The alignment of Sit (Dm Sit) with the conserved region of a family of membrane-bound acyltransferases [37]. An arrow marks the position of an invariant histidine proposed to be an active site residue. Sequences shown are from human cholesterol acyltransferase (Hs ACAT), mouse diacylglycerol O-acyltransferase (Mm DGAT), Staphylococcus DltB (Sa dltB), Treponema Alg I (Tp algI), and fly Porcupine (Dm Porc).

males. Larvae were heat shocked as described above. FRT80B, sit^{T392}/FRT80B, P(ovo^{D}); *hs*FLP122/+ females were crossed to sit^{T802}/TM6B males to generate the embryos shown. Half of the embryos hatched and developed into larvae with the TM6B, *Tb* chromosome.

Immunohistochemistry

Eye and wing discs were stained with antibodies and X-gal as described [6, 42]. The antibodies that were used included rat anti-Elav (1:1), rabbit anti-Ato (1:5000) [21], rabbit anti-β-galactosidase (Cappel; 1:5000), rat anti-Ci (1:1) [26], mouse anti- Wg (1:10) [29], and rabbit anti-Hh-N (1:2000) [6]. For Western blotting, imaginal discs and brains from wild-type and *sit* mutant third instar larvae were homogenized directly in protein gel loading buffer. Western blotting with rabbit anti-Hh-N was performed as described [6].

Molecular biology

sit was mapped using site-directed male recombination [36] with the following P element insertions: *l(3)06803*, *EP(3)3109*, *l(3)S103012*, *l(3)j5c2*, *l(3)S049217*, and *l(3)09143*. Between one and four recombinants were obtained for each P element. Insertion site information was not available for *l(3)S103012* or *l(3)S049217*, so in these cases, flanking DNA was obtained by inverse PCR, sequenced, and compared to the *Drosophila* genome sequence. *l(3)S103012* is inserted at position 95524, and *l(3)S049217* is inserted at position 186052 in clone AE003477. Genomic DNA was prepared from larvae homozygous for FRT80B, sit^{T392} or FRT80B, sit^{T802} or from adults homozygous for FRT80B, and the full open reading frame of CG11495 was amplified by PCR as a 1.6-kb fragment. sit^{T392} had a CAG to TAG change converting Q287 to a stop codon. sit^{T802} had a deletion of the underlined 10 bp: GTGCAT CAATGTGATGCAAAAGAATGT, causing a frame shift at N156, followed by 17 extraneous amino acids and a stop codon. Both changes were confirmed in a second independent PCR reaction, and neither was present in DNA from the FRT80B flies. A PCR fragment covering the 5′-most 1.1 kb of this region was used to screen the LD embryonic cDNA library obtained from the Berkeley *Drosophila* genome project. Three cDNA clones were obtained (GenBank accession number AF393157); the two longest initiated 3 bp upstream of the initiator ATG codon. This codon occurs 185 bp after the 3′ end of the previous predicted gene, CG15812. The full-length cDNA in pBluescript was used as a template to make sense and antisense probes for in situ hybridization, which was performed as described [43]. PHDhtm and TopPred2 were used to predict transmembrane domains, and blastp and ALIGN were used to align the *Drosophila* and human sequences. The alignment in Figure 4c is based on [37].

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