

Drosophila Gp150 is required for early ommatidial development through modulation of Notch signaling

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Cellular signaling activities must be tightly regulated for proper cell fate control and tissue morphogenesis. Here we report that the *Drosophila* leucine-rich repeat transmembrane glycoprotein Gp150 is required for viability, fertility and development of the eye, wing and sensory organs. In the eye, Gp150 plays a critical role in regulating early ommatidial formation. Gp150 is highly expressed in cells of the morphogenetic furrow (MF) region, where it accumulates exclusively in intracellular vesicles in an endocytosis-independent manner. Loss of *gp150* function causes defects in the refinement of photoreceptor R8 cells and recruitment of other cells, which leads to the formation of aberrant ommatidia. Genetic analyses suggest that Gp150 functions to modulate Notch signaling. Consistent with this notion, Gp150 is co-localized with Delta in intracellular vesicles in cells within the MF region and loss of *gp150* function causes accumulation of intracellular Delta protein. Therefore, Gp150 might function in intracellular vesicles to modulate Delta–Notch signaling for cell fate control and tissue morphogenesis.

Keywords: Delta/*Drosophila* eye/leucine-rich repeat/Notch

Introduction

Development of multicellular organisms requires not only precise cell fate specification, but also proper patterning of differentiating cells. The *Drosophila* compound eye provides a model system to address how cells are specified and organized. During the third instar larval stage, a dorso-ventral indentation called the morphogenetic furrow (MF) moves from the posterior of the eye tissue to the anterior, and formation of ommatidial clusters is initiated in areas immediately posterior to the MF (for review see Wolff and Ready, 1993). Expression of the proneural basic helix–loop–helix (bHLH) *atonal* (*ato*) gene is first induced by Hedgehog signaling in a stripe of cells just anterior to the MF (for review see Heberlein and Moses, 1995; Treisman and Heberlein, 1998). Within the MF, *ato* expression is restricted to regularly spaced proneural clusters of cells. Only one cell from each proneural cluster continues to express *ato* and becomes the R8 photoreceptor (R), which then initiates further recruitment of other R cells for ommatidial construction (Jarman *et al.*, 1994, 1995; Baker

et al., 1996; Dokucu *et al.*, 1996; Baonza *et al.*, 2001). Notch (N) signaling initially plays a positive role in establishing high levels of *ato* in cells anterior to the MF (Baker and Yu, 1997; Li and Baker, 2001) by down-regulating Hairy (H) and Extramacrochaetae (Emc), two repressors of *ato* (Baonza and Freeman, 2001). Subsequently, N-mediated lateral inhibition is required for the refinement of *ato* expression to regularly spaced individual R8 precursor cells (Parks *et al.*, 1995; Baker *et al.*, 1996; Dokucu *et al.*, 1996). The *Drosophila* homolog of the vertebrate epidermal growth factor receptor (DER) functions to regulate R8 spacing as well. DER signaling acts non-autonomously to inhibit *ato* expression in cells anterior and lateral to the proneural clusters to generate regularly spaced proneural clusters (Chen and Chien, 1999; Baonza *et al.*, 2001). Also, a glycoprotein Scabrous (Sca) might be responsible for anterior and lateral repression of Ato, as Sca is produced in proneural clusters and can be secreted (Baker and Zitron, 1995; Lee *et al.*, 1996). Sca associates with N *in vivo* and can stabilize N proteins at the cell surface (Powell *et al.*, 2001).

In precursor cells located more posterior to the MF, N signaling is required for restricting cellular competence to respond to receptor tyrosine kinase (RTK)-mediated inductive signaling for R cell specification. Supporting this idea, several E(spl) proteins are prominently expressed in the basally located nuclei of the precursor cells (Baker *et al.*, 1996). A deletion of a subset or all of the bHLH genes in the E(spl) complex results in a severe neurogenic phenotype in the posterior region of eye discs (Treisman *et al.*, 1997; Ligoxygakis *et al.*, 1998). Thus, coordinated action of inductive RTK signaling (for review see Freeman, 1997) and N-mediated lateral inhibition (for review see Artavanis-Tsakonas *et al.*, 1999; Mumm and Kopan, 2000) is critical for the sequential recruitment of R cells into the developing ommatidia.

Here we report the identification and isolation of loss-of-function mutations in the *Drosophila gp150* gene, which encodes a leucine-rich repeat (LRR) protein that is required for viability, fertility and proper development of the eye, wing and sensory organs. In the eye, removal of *gp150* function causes defects in the refinement of R8 cells and recruitment of other cells, which leads to the formation of fused ommatidia, as well as ommatidia containing too many or too few R cells. We show that Gp150 is expressed at high levels in the MF region, which is consistent with a role of *gp150* in early ommatidial development. Moreover, genetic analyses suggested that Gp150 functions to modulate Delta (Dl)–N signaling, and immunostaining experiments showed that Gp150 is co-localized with Dl in intracellular vesicles in cells within the MF region. Gp150 might be involved in facilitating lysosomal delivery of the Dl protein and/or Dl transport to the plasma membrane,

as loss of *gp150* function causes accumulation of intracellular DI protein. Gp150 appears to be a resident protein of intracellular vesicles and its localization is not affected in endocytosis-defective cells. Based on these observations, we propose that Gp150 might function in subcellular vesicles to control appropriate intracellular levels of DI to modulate N signaling.

Results

Identification and isolation of loss-of-function mutations in the *Drosophila gp150* gene, which encodes a LRR transmembrane protein

Flies homozygous for two presumably P transposon-induced semi-lethal mutations, l(2)k11107 and l(2)k11120 (Torok *et al.*, 1993), exhibit reduced fertility and abnormal bristle, wing and eye phenotypes. These two allelic recessive mutations were meiotically mapped to the tip of the right arm of the second chromosome. Deficiency mapping further defines the locus of these mutations to the 58D1-5 region. However, no wild-type revertants were recovered by P-element excision (data not shown). Thus, these alleles could have been caused by an imprecise excision of a P-element initially inserted at the locus. To facilitate molecular genetic analysis of this gene, we generated new alleles through ethyl methane sulfonate (EMS) mutagenesis and P-element local hopping (see

Materials and methods). Genomic sequences flanking the insertion sites in two of the newly isolated P alleles, P2 and P8, were cloned and used to isolate cDNA clones from a larval eye disc cDNA library. Sequence analysis of full-length cDNAs demonstrated that these cDNA molecules encode a polypeptide identical to the LRR transmembrane glycoprotein Gp150 (Tian and Zinn, 1994).

Our sequence analysis, together with data derived from the *Drosophila* genome project, indicates that the *gp150* gene consists of six exons and five introns (Figure 1A). The *gp150* open reading frame (ORF) is restricted to the last four exons, which encode a polypeptide of 1051 amino acids. The following evidence demonstrates that mutant alleles of *gp150* have been identified. First, in the P8 allele, a P-element is inserted within the second intron of the *gp150* transcription unit, 53 bp downstream of the second exon (Figure 1A). The P8 mutant eye discs exhibited a reduction of *gp150* expression, as shown by western blotting (Figure 1B) and immunostaining (data not shown). These experiments revealed that P8 is a partial loss-of-function mutation. Furthermore, mutant phenotypes in four out of six newly isolated P alleles can be frequently reverted to wild type by P-element excision (see Materials and methods), indicating that these mutations were indeed caused by P-element insertions. Secondly, a single base change of G to A was found in codon 94 for tryptophan in an EMS allele (*gp150*⁴), which results in a nonsense mutation. Thirdly, western blot and immunostaining analyses revealed that the level of Gp150 is either reduced or eliminated by the mutations (Figures 1B and 5B). Lastly, the mutant eye phenotypes can be effectively rescued by overexpression of a wild-type *gp150* gene in the developing eye (Figure 3E and F). These loss-of-function mutations were used in this study to reveal a role of *gp150* in eye development.

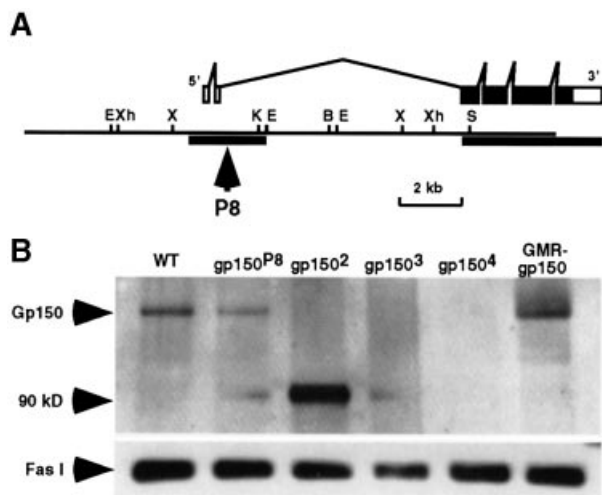


Fig. 1. Identification of loss-of-function alleles of the *gp150* gene. (A) The *gp150* gene consists of six exons and five introns with the complete coding sequence restricted within a 4 kb genomic region. The filled boxes represent a complete 3156 bp ORF. Sequences of two genomic regions indicated by the thick horizontal lines were determined to illustrate intron–exon structures of the *gp150* transcriptional unit. A PCR method was used to obtain the downstream genomic sequence of *gp150* that was not included in the genomic clone. In P8, a P transposon was inserted in the second intron of *gp150*. Restriction enzymes used for mapping include *Bam*HI (B), *Eco*RI (E), *Kpn*I (K), *Sal*I (S), *Xba*I (X) and *Xho*I (Xh). (B) A western blot was probed with a Gp150 antibody that was made against the N-terminal region (amino acids 5–192) of Gp150. Since no Gp150 protein was detectable in *gp150*², *gp150*³ and *gp150*⁴ mutants, these alleles can be considered as molecular nulls. The *gp150*² mutation results in the production of a non-functional 90 kDa protein. This is based on observations that *gp150*² is recessive and the mutant phenotypes caused by *gp150*² are similar to that of *gp150*³ and *gp150*⁴. The 70 kDa Fas I protein served as an internal control and was detected by anti-Fas I (MAb6D8) antibody in all lanes.

gp150 is required for viability, fertility and normal development of the wing and sensory organs

Loss of *gp150* function results in a strong reduction in viability. For instance, >60% of *gp150*²/*gp150*³ flies fail to develop to the adult stage, with most of them dying during pupal development (Table I). *gp150* mutant females are sterile as they fail to lay eggs and the fertility of *gp150* mutant males is also strongly reduced (data not shown). In *gp150*⁻ wings, the size and overall morphology appear normal (Figure 2B). However, closer examination revealed that some dorsal margin bristles are missing or duplicated and ectopic bristles can be found in the distal region of the second vein (Figure 2D). Moreover, the distal end of the third vein is enlarged to form a ‘Delta-like’ structure (Figure 2F).

Table I. Gp150 is required for viability

	1	2	3	4	<i>Df(2R)02311</i>
1		61 (<i>n</i> = 633)	75 (<i>n</i> = 747)	73 (<i>n</i> = 221)	61 (<i>n</i> = 334)
2			61 (<i>n</i> = 526)	43 (<i>n</i> = 319)	46 (<i>n</i> = 319)
3				93 (<i>n</i> = 476)	63 (<i>n</i> = 331)
4					62 (<i>n</i> = 355)

1, 2, 3 and 4 correspond to *gp150*¹, *gp150*², *gp150*³ and *gp150*⁴, respectively. For example, from a cross between *gp150*²/*CyO* and *gp150*³/*CyO*, 61% of the predicted *gp150*²/*gp150*³ mutants fail to develop to the adult stage. *n* = total number of adult flies scored.

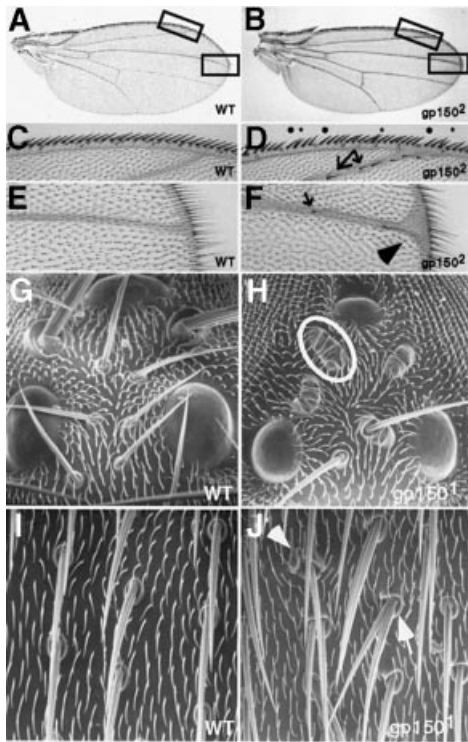


Fig. 2. Loss of *gp150* function results in defective wing and sensory organs. Wild-type (A, C and E) and *gp150*² mutant (B, D and F) wings are shown. (C) and (E) are enlarged from boxed areas in (A). (D) and (F) are enlarged from the boxed areas in (B). Some bristles are missing (dots) or duplicated (asterisks) in the wing margin (D). Arrows point to some ectopic bristles in the second and third veins (D and F) and the arrowhead identifies an enlarged 'Delta-like' vein structure (F). SEM images of wild-type (G and I) and *gp150*¹ mutant flies (H and J) are shown to illustrate phenotypes in ocellar bristles (G and H) and sensory organs over the notum (I and J). Multiple socket cells are often developed in sensory organs (circled in H and indicated by an arrowhead in J). The external shaft is often missing or duplicated (indicated by an arrow in J). Moreover, there are cases where both socket and shaft cells are absent in some sensory organs (H). All other *gp150* alleles exhibit similar phenotypes in the wing and sensory organs.

During normal bristle development, a single sensory organ precursor (SOP) cell divides to produce two daughter cells, IIa and IIb, which divide and generate a trichogen (shaft) and a tormogen (socket), a neuron, a thecogen (sheath) and a glial cell, respectively (for review see Jan and Jan, 1995; Gho *et al.*, 1999). In *gp150* mutants, sensory organ development is defective in ocellar, humeral, scutellar and sensory bristles over the notum and dorsal abdomen (Figure 2H and J). In all of the *gp150*¹ mutant flies, one or more ocellars were found to contain multiple socket cells with the external shaft missing (Figure 2H). Such mutant phenotypes implicate cell fate transformation from IIb to IIa and shaft to socket. Interestingly, external cells (socket and shaft) of some sensory organs appeared to be missing (Figure 2H), suggesting a possible cell fate transformation from IIa to IIb. Such mutant phenotypes were observed in other sensory organs as well. For instance, 68% ($n = 31$) of the anterior scutellars in *gp150*²/*gp150*³ flies exhibited no shaft and no socket, 23% with double socket, 3% with one shaft/two sockets and 3% with two shafts/one socket

phenotypes. In conclusion, the *gp150* function is required for proper development of several distinct tissue types.

Loss of *gp150* function results in defective ommatidial development

We have focused on analyzing *gp150* function using the eye system. The wild-type compound eye of *Drosophila melanogaster* represents an organization of 800 precisely arrayed unit eyes called ommatidia (Figure 3A). Each ommatidium contains 12 accessory cells and eight R cells arranged in a trapezoidal configuration (Figure 3B). Compared with the wild type, *gp150* mutant eyes are rough. Many ommatidia are irregular in shape and size and contain either too many or too few R cells. Also, fused ommatidia are often visible (Figure 3C and D). These results indicate that *gp150* is required for normal ommatidial development. Clonal analysis revealed essentially the same mutant phenotypes as those observed in *gp150* homozygous mutants (data not shown).

To reveal the developmental basis of the eye defects, *gp150* mutant third instar eye discs were examined. In wild-type eye discs, phalloidin staining outlines apical cell surfaces and highlights the constricted cells within the MF as well as the cells within the evenly spaced developing ommatidial clusters (Figure 4A). In *gp150* mutants, ommatidial clusters are often irregularly spaced and the MF appears broader as the cells within the region are no longer constricted in a narrow stripe (Figure 4B). To elucidate early developmental defects that might lead to the aberrant ommatidial organization, *ato* expression was examined. *ato* is essential for R8 specification (Jarman *et al.*, 1994) and is initially expressed in cells anterior to the MF. Within the MF, *ato* expression is first restricted to proneural groups of cells and then to individual R8 precursor cells (Figure 4C; Jarman *et al.*, 1995). In *gp150* mutant eye discs, Ato-positive proneural clusters are abnormally patterned and multiple R8 precursor cells are often closely located (Figure 4D). About 10% ($n = 72$) of the ommatidia contain multiple Ato-positive R8 precursors that become R8 cells, as inferred from their expression of R8-specific Bride-of-sevenless (Boss) protein (Figure 4F; Kramer *et al.*, 1991). To examine how ommatidial assembly can be affected by *gp150* mutations, a neural-specific nuclear marker, Elav (Robinow and White, 1991), was used to highlight all R cells. In the wild type, developing ommatidia are evenly patterned with precursor cells sequentially recruited into ommatidia clusters (Figure 4G). In contrast, ommatidia are irregularly organized in *gp150* mutant eye discs. Some oversized ommatidia are seen at early stages of ommatidia assembly (Figure 4H). Thus, recruitment of R cells into the clusters appears to occur prematurely in some clusters.

To address whether accumulation of oversized ommatidia close to the MF is due to defects in furrow progression, expression of *decapentaplegic* (*dpp*) and a bHLH gene *hairy* (*h*) was examined, since both of them are involved in regulating furrow progression (Brown *et al.*, 1995; for review see Treisman and Heberlein, 1998). No discernible effect on either *h* expression anterior to the furrow or *dpp* expression within the MF was detected in *gp150* mutants (Figure 4G and H and data not shown). Moreover, unlike mutations that disrupt furrow initiation and progression (Heberlein *et al.*, 1993; Ma

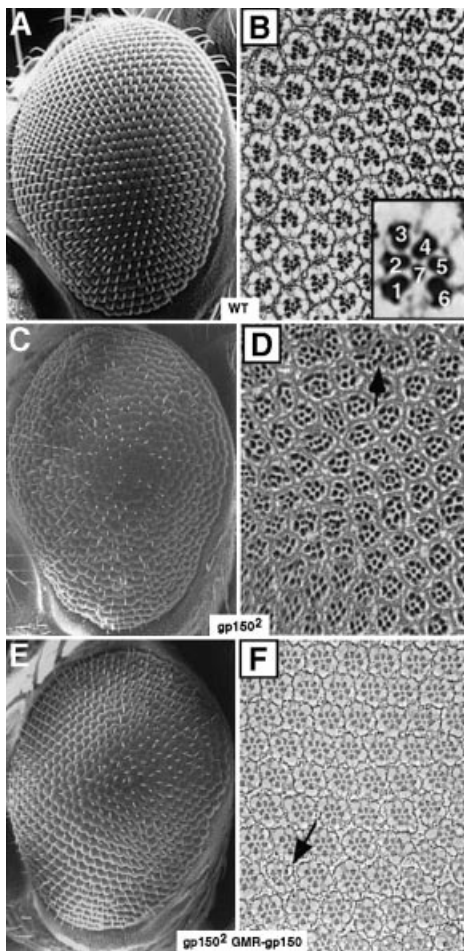


Fig. 3. Loss of *gp150* function causes defective eye development. SEM images (A, C and E) and tangential sections (B, D and F) of adult eyes are presented. (A and B) Wild type. The inset highlights seven R cells arranged in a trapezoidal configuration in each ommatidium and the rhabdomere of R8 cannot be seen in this apical section. (C and D) *gp150*² mutants. About 35% ($n = 522$) of the ommatidia contain either too many or too few R cells. The arrow indicates a fused ommatidium. At basal levels, ommatidia with multiple R8-like cells were observed (data not shown). Other *gp150* alleles exhibit similar phenotypes. (E and F) In *gp150*² *GMR-gp150* flies, overexpression of *gp150* in the eye effectively rescues the mutant eye phenotype, with >93% ($n = 368$) of the ommatidia containing a normal complement of R cells. The arrow indicates a mutant ommatidium occasionally seen in this genotype. Anterior is to the left, except (D), in which anterior might be to the top.

et al., 1993), *gp150* mutations do not cause an apparent size reduction of the eye (Figure 3C). These results suggest that *gp150* mutations do not disrupt initiation or progression of the MF.

To find out whether cells recruited at later stages of ommatidial assembly are affected by *gp150* mutations, expression of a homeodomain protein BarH1 was examined. In wild-type eye discs, BarH1 is specifically expressed in R1 and R6 cells (Figure 4I; Higashijima *et al.*, 1992). In the mutant, most R1/R6 cells are irregularly positioned (Figure 4J). Their abnormal arrangement further indicates defective ommatidial organization. Moreover, some ommatidia contain additional or fewer R1/R6 cells (Figure 4J) and cone cells (Figure 4L). Thus, phenotypic analysis of *gp150* mutant eye discs indicates

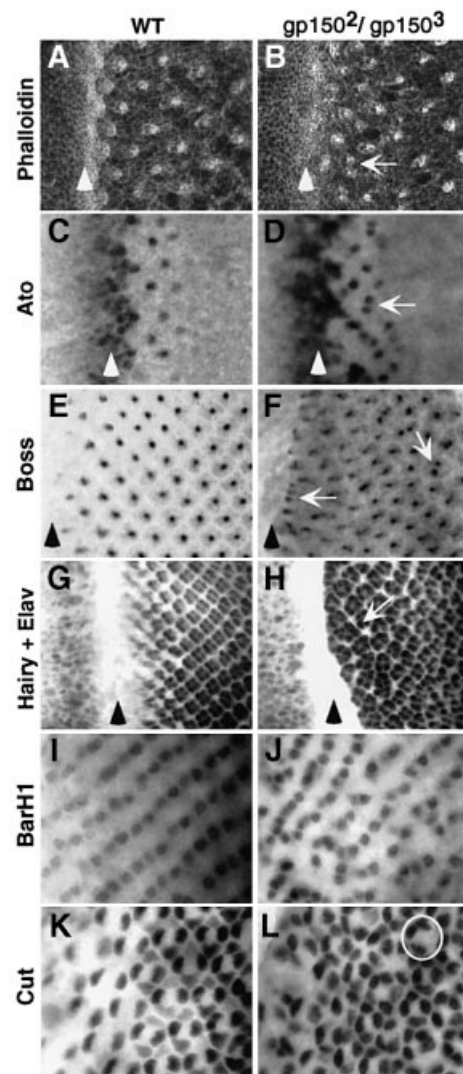


Fig. 4. Loss of *gp150* function causes defects in early ommatidial development. Wild-type (A, C, E, G, I and K) and *gp150*²/*gp150*³ mutant (B, D, F, H, J and L) third instar eye discs are shown. (A and B) Phalloidin staining. (C and D) Anti-Ato antibody staining. Arrows in (B and D) point to closely located ommatidia. (E and F) Anti-Boss antibody staining. Arrows indicate ommatidia containing more than one Boss-positive R8 cells. (G and H) Double staining for Hairy and Elav. The arrow points to an oversized ommatidium near the furrow. (I and J) Anti-BarH1 antibody staining. (K and L) Anti-Cut antibody staining. The circle indicates an ommatidium missing a cone cell. Arrowheads identify the location of the MF in (A–H). Anterior is to the left in all panels.

that *gp150* plays a critical role in regulating ommatidial patterning and assembly in the developing eye.

***Gp150* is expressed at high levels within cells of the MF region and is localized to intracellular vesicles**

To characterize the normal pattern of Gp150 expression in the developing eye, Gp150 antibodies were generated and used for immunostaining experiments. Specificity of the Gp150 antibody was confirmed by the absence of staining in *gp150*⁴ eye discs (Figure 5B) and the increased staining in *GMR-gp150* eye discs (Figure 5C). In wild-type eye discs, cells immediately anterior to and within the furrow

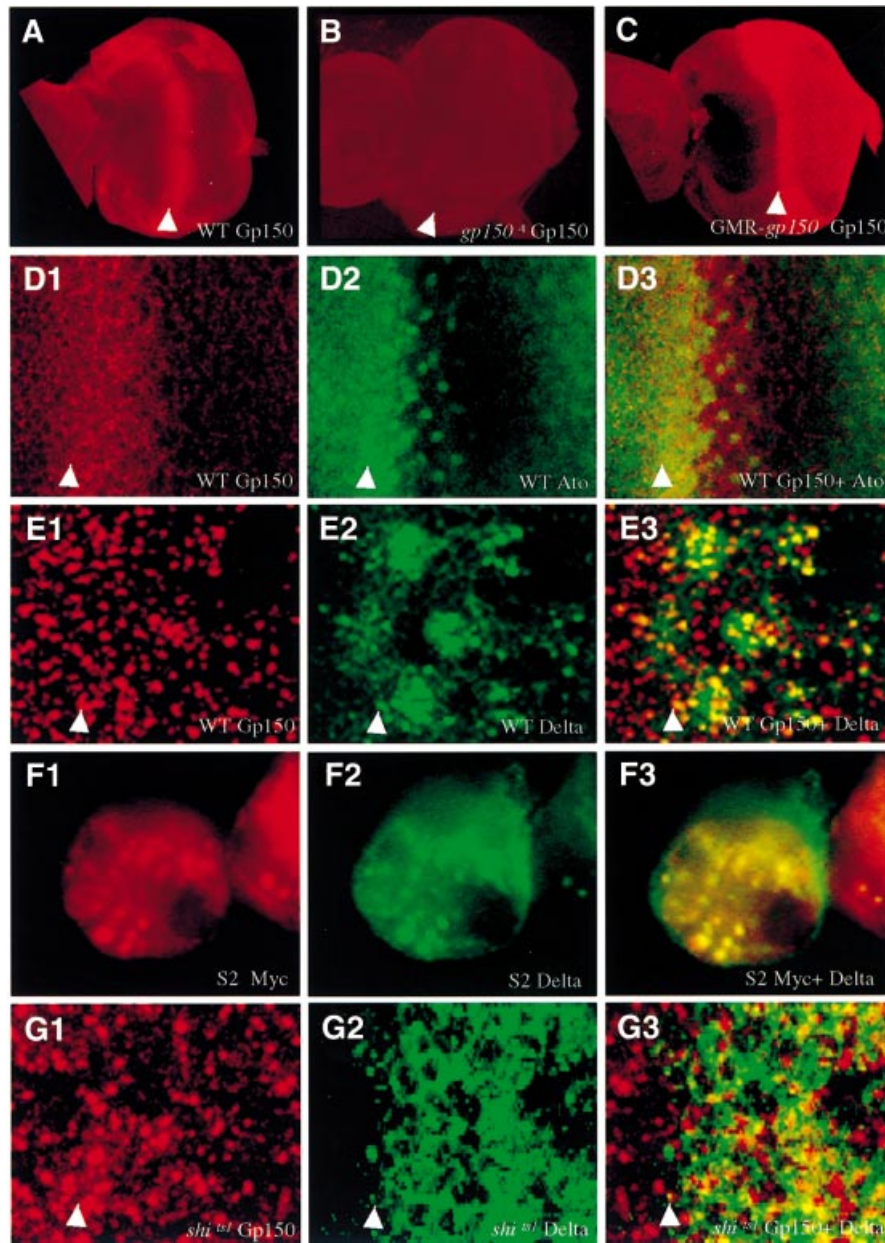


Fig. 5. *gp150* is expressed at high levels in the MF region in the developing eye. (A) High levels of Gp150 protein were detected in the MF region of wild-type third instar eye discs. Posterior to the furrow, relatively lower levels of Gp150 can be detected around each individual ommatidium. (B) Gp150 expression was not detectable in *gp150⁴* eye discs. (C) High levels of Gp150 expression were detected in all cells behind the furrow in *GMR-gp150* eye discs. (D1–3) Double-staining reveals Gp150 (red, D1) and Ato (green, D2) expression in eye discs. A superimposed image from (D1) and (D2) is shown in (D3). (E1–3) Gp150 and Dl proteins were shown to co-localize in subcellular vesicles in the furrow region. (F1–3) In S2 cells co-transfected with *pmt-gp150-myc* and *pmt-Dl*, Gp150 and Dl were also shown to co-localize in subcellular vesicles. (G1–3) Heat-treated *shi^{ts1}* mutant third instar eye discs were double stained with anti-Gp150 (red in G1) and anti-Dl (green in G2) antibodies. A superimposed image is shown in (G3). Arrowheads indicate the location of the MF, and anterior is to the left in all panels.

region express high levels of Gp150 protein (Figure 5A and D1). Gp150 proteins accumulate in many small vesicles throughout the apical–basal axis in the furrow region. The area with high levels of Gp150 mostly overlaps with cells expressing the proneural gene *ato* (Figure 5D1–3). This elevated expression of Gp150 in the furrow region is consistent with the notion that Gp150 is required to regulate early ommatidial development. Posterior to the furrow, lower levels of Gp150 were detected around each individual ommatidium (Figure 5A).

The Gp150 protein was not detectable in the nucleus or at the plasma membrane of the eye disc cells.

To examine how Gp150 might distribute in subcellular compartments, we used a Myc-tagged non-functional Fringe protein as a Golgi-specific marker (Munro and Freeman, 2000). Partial overlap between Gp150- and Fringe-positive vesicles was observed in both *sca-Gal4/UAS-fringe^{ADD-myc}* and *GMR-Gal4/UAS-fringe^{ADD-myc}* eye discs (data not shown). Using a green fluorescent protein (GFP)-tagged *Drosophila* Rab7 protein as a

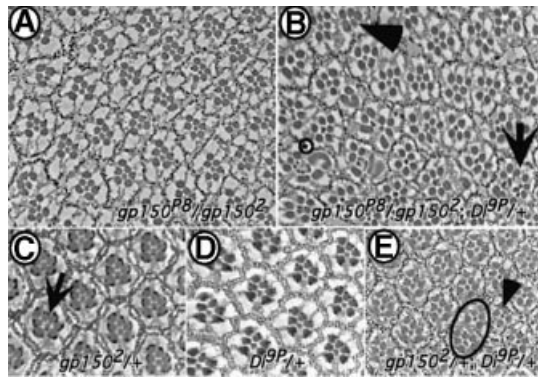


Fig. 6. Genetic interactions between *gp150* and *Delta*. Tangential sections of adult eyes are presented. (A) The *gp150^{P8}/gp150²* eye exhibits ~19% ($n = 578$) mutant ommatidia. (B) Reduction of *Dl* function strongly enhances the *gp150* mutant phenotypes. Up to 68% ($n = 544$) abnormal ommatidia, which contain more R cells (arrow), fewer R cells (arrowhead) or apparently fused ommatidial units, were found in *gp150^{P8}/gp150²; Dl^{9P}/+* flies. Often rhabdomeres exhibit abnormal morphology (circled asterisk). (C) *gp150²/+*. The arrow points to an occasionally observed aberrant ommatidium. (D) *Dl^{9P}/+* flies, no mutant ommatidia can be found. (E) *gp150²/+; Dl^{9P}/+*. The circle indicates a fused ommatidium and the arrowhead points to an ommatidium missing an outer R cell.

marker for late endosomes (Entchev *et al.*, 2000), we also observed that Gp150 can be partially localized to endosomes (data not shown). Thus, these results support the idea that Gp150 is involved in regulating vesicle trafficking.

gp150* genetically interacts with *Dl* and *N

The *gp150* mutant eye phenotypes closely mimic those caused by defective *Dl*-*N* signaling (Cagan and Ready, 1989; Baker *et al.*, 1990; Parks *et al.*, 1995). Thus, Gp150 might be functionally connected to the *N* pathway. To investigate this possibility, two assays were devised to test genetic interactions between *gp150* and *Dl* and *N*. In a genetic sensitization assay, the *gp150* function is partially removed in *gp150^{P8}/gp150²* flies, which allows us to monitor dominant effects of mutations in other genes on *gp150* mutant eye phenotypes. Reduction of *Dl* function strongly enhanced the *gp150* mutant eye phenotypes (Figure 6A and B). There was an ~2- to 3-fold increase in the number of mutant ommatidia in *gp150^{P8}/gp150²; Dl/+* flies compared with the control (Table II). Although not as effective as *Dl*, *N* mutations exhibited a similar effect (Table II). These results suggest that Gp150 functions together with *Dl* and *N* to regulate ommatidial development. Using the same assay, mutations such as *rl^{S-135}*, *Ras1^{e1B}* and *Ras1^{e2F}* did not effectively alter the *gp150* mutant eye phenotypes (data not shown). Both *Ras1* and a MAP kinase, which is encoded by the *rolled* (*rl*) gene, are key components of the RTK pathway.

Loss-of-function alleles of *gp150* exhibit a very weak dominant eye phenotype as a small number of aberrant ommatidia were found in *gp150* heterozygotes (Figure 6C; Table II). This is most likely due to haplo-insufficiency since a deficiency chromosome, in which *gp150* is completely deleted, exhibits the same phenotype (Table II). In this second assay, *Dl* mutations also dominantly enhanced *gp150* mutant eye phenotypes, as

Table II. *gp150* genetically interacts with Notch and Delta

Genotype	No. of ommatidia examined	% mutant
<i>gp150^{P8}/gp150²</i>	578	19.2
<i>N^{55el1/+}; gp150^{P8}/gp150²</i>	629	42.3
<i>gp150^{P8}/gp150²; Dl^{9P}/+</i>	544	67.6
<i>gp150^{P8}/gp150²; Dl^{6B}/+</i>	460	44.6
<i>Df(2R)02311/+</i>	645	0.15
<i>gp150²/+</i>	1051	0.19
<i>N^{55el1/+}</i>	599	0
<i>N^{55el1/+}; gp150²/+</i>	769	0.65
<i>Dl^{9P}/+</i>	655	0
<i>gp150²/+; Dl^{9P}/+</i>	852	2.70
<i>Dl^{6B}/+</i>	534	0
<i>gp150²/+; Dl^{6B}/+</i>	558	0.18
<i>Dl^{6B}/+ (29°C)</i>	539	0.37
<i>gp150²/+; Dl^{6B}/+ (29°C)</i>	330	1.52

For each genotype, 5–9 adult eyes were sectioned and scored. Flies were cultured at 25°C, unless indicated otherwise. Ommatidia that were fused or contained too many or too few rhabdomeres were counted as mutant.

there was a >10-fold increase in the number of mutant ommatidia in *gp150²/+; Dl^{9P}/+* flies (Figure 6C–E; Table II). Compared with *Dl*, *N* mutations exhibited a weaker enhancing effect (Table II). Altogether, these genetic data suggest that Gp150 is involved in modulating *Dl*-*N* signaling in the eye.

***Dl* is co-localized with *Gp150* in intracellular vesicles within cells of the MF region**

To begin to investigate how Gp150 might act to modulate *N* signaling, we examined whether *Dl* and Gp150 proteins were co-localized. Cell surface and intracellular expression of *Dl* within cells of the developing eye has been characterized previously (Parks *et al.*, 1995; Baker and Yu, 1998). *Dl* protein accumulates to higher levels in endosomal vesicles than on the plasma membrane. Some of these vesicles correspond to multi-vesicular bodies (MVBs) (Parks *et al.*, 1995). Double-labeling experiments showed that *Dl* was co-localized with Gp150 in intracellular vesicles of cells within the furrow region (Figure 5E1–3). Moreover, Gp150 and *Dl* proteins were found to be co-localized in 60 out of 66 subcellular vesicles examined in S2 cells that were positive for both Gp150 and *Dl* expression (Figure 5F1–3). These results suggest that Gp150 may act in late endosomes to modulate the level or activity of *Dl* protein in the morphogenetic furrow region. Moreover, it is also possible that Gp150 is co-localized with *Dl* in secretory vesicles or recycling endosomes to facilitate *Dl* presentation to the plasma membrane.

***Gp150* accumulation in intracellular vesicles is independent of Dynamin GTPase-mediated endocytosis**

The accumulation of *Dl* in intracellular vesicles is dependent on Dynamin-mediated endocytosis, which is critical for *N* signaling (Parks *et al.*, 1995, 2000). The *Drosophila* Dynamin GTPase is encoded by the *shibire* (*shi*) gene, which is required for endocytosis (Chen *et al.*,

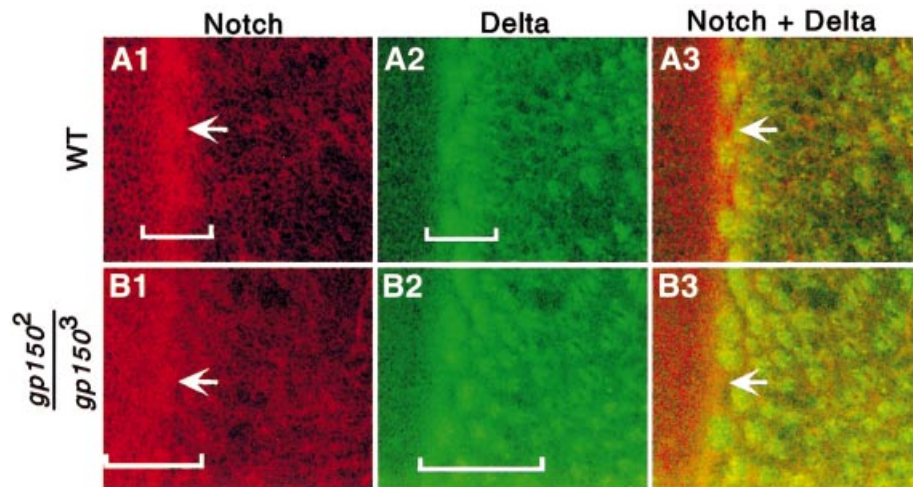


Fig. 7. Expression of Delta and Notch in wild-type and *gp150* mutant eye discs. Wild-type (A1–3) and *gp150*²/*gp150*³ mutant (B1–3) third instar eye discs were stained with anti-N (red in A1 and B1) and anti-Dl (green in A2 and B2) antibodies. Superimposed images are shown in (A3 and B3). Brackets indicate areas that show relatively higher N or Dl levels in the MF region. Arrows point to N-positive cells in between Dl-positive clusters in the MF. Anterior is to the left in all panels.

1991; Van der Blik and Meyerowitz, 1991). In *shi*^{ts1} mutants, Dl proteins fail to be endocytosed and, therefore, accumulate at the plasma membrane (compare Figure 5E2 with G2; Parks *et al.*, 1995). To test whether the accumulation of Gp150 in vesicles is also endocytosis dependent, expression of Gp150 was examined in heat-treated *shi*^{ts1} eye discs. Unlike Dl, vesicle localization of Gp150 was not apparently altered when *shi* function was removed (compare Figure 5E1 with G1). Thus, Gp150 proteins accumulate in intracellular vesicles in an endocytosis-independent manner.

***gp150* mutations affect the expression of Dl and N proteins in the MF region**

To examine whether loss of *gp150* function affects levels and patterns of N and Dl expression in the developing eye, N and Dl antibodies were used for immunostaining experiments. In wild-type eye discs, N is ubiquitously expressed at the cell surface, with some N proteins being detected in subcellular vesicles (Figure 7A1). Cells in the MF accumulate relatively high levels of Notch. Interestingly, cells between Dl-positive clusters in the furrow exhibit even higher levels of N protein (Figure 7A1–3). N expression in the *gp150* mutant appeared to be slightly expanded in the MF region and the elevated expression of N in cells between Dl-positive clusters is less apparent (Figure 7B1–3). Posterior to the furrow, high levels of Dl in ommatidial clusters persisted for ~3–4 rows both at the cell surface and in endosomal vesicles in wild-type eye discs (Figure 7A2 and B3; Parks *et al.*, 1995; Baker and Yu, 1998). However, in *gp150* mutants, high levels of Dl were found in clusters distributed over 6–7 rows posterior to the furrow (Figure 7B2 and B3). In addition, regular spacing between Dl-positive clusters was disrupted. Thus, loss of *gp150* function alters the expression pattern of Dl and N proteins and, in particular, the level of intracellular Dl appears to be negatively regulated by Gp150 in the furrow region.

Discussion

LRR proteins exist in organisms ranging from plants to metazoan animals and constitute an evolutionarily conserved superfamily (for review see Kobe and Deisenhofer, 1994; Kajava, 1998). These proteins are found in different subcellular locations and function in a wide variety of processes such as cell adhesion and signal transduction. Based on our expression analysis using eye discs, Gp150 is exclusively located in intracellular vesicles, some of which are late endosomes and the Golgi apparatus. As Gp150 accumulation in intracellular vesicles does not depend on the Dynamin-mediated endocytic pathway, Gp150 appears to accumulate in the vesicles through an intracellular trafficking route.

A role of Gp150 in modulating Dl–N signaling during early ommatidial development

The development of hundreds of precisely organized ommatidia in the *Drosophila* compound eye begins with the specification and regular spacing of R8 cells, which become founder cells required for the sequential recruitment of other cell types. Our phenotypic analysis demonstrated that Gp150 plays a critical role during ommatidial development. In the absence of *gp150* function, both the selection and patterning of the R8 cells become aberrant and a small number of ectopic R8 cells appear to be specified. The *gp150* mutant eye phenotypes mimic what occurs in loss-of-function mutants of *Dl* and *N* (Cagan and Ready, 1989; Baker *et al.*, 1990; Parks *et al.*, 1995), suggesting that Gp150 might facilitate Dl–N signaling. Supporting this hypothesis, the reduction of *Dl* or *N* function dominantly enhanced the *gp150* mutant eye phenotypes.

Clonal analysis was carried out to reveal in which cells the function of Gp150 is required. By examining phenotypically normal but genetically mosaic ommatidia, we found that *gp150* could be either wild type or mutant in all R cells (R1–R8; data not shown). Therefore, the

function of *gp150* does not appear to be required in R cells for normal ommatidial development. Given that R cells are signal-sending cells, which prevent neural differentiation in neighboring precursor cells, this result is consistent with a view that *gp150* might be required in the precursor cells, which are presumably DI–N signal-receiving cells.

How might Gp150 modulate DI–N signaling? So far, Gp150 is the only protein known to co-localize with DI in intracellular vesicles. In a simple model, Gp150 might facilitate DI presentation at the cell surface. Gp150 might also promote fusion between DI-positive MVBs and lysosomes so that the intracellular levels of DI can be reduced. The latter mechanism might be used to reduce the autonomous inhibitory effect of DI on N signaling in the signal-receiving cells. In the absence of *gp150* function, the levels of intracellular DI increase, which autonomously blocks the N pathway in N signal-receiving cells and possibly makes these cells more responsive to instructive signals for R cell specification and differentiation, thus resulting in ommatidia with too many R cells. In tissues such as the wing and sensory organs, DI has been shown to autonomously inhibit N signaling in a signal-receiving cell (Jacobsen *et al.*, 1998; de Celis and Bray, 2000). In some cases, increased intracellular levels of DI might allow some DI to be presented at the cell surface to initiate N signaling on adjacent cells. This would increase lateral inhibition and result in the formation of ommatidia with fewer R cells. The fewer R cell phenotype could also be due to an insufficient number of competent retinal precursor cells that are available for the competing ommatidial clusters during R cell recruitment in *gp150* mutants. In any event, we do not exclude the possibility that Gp150 might use other mechanisms to modulate DI–N signaling. For instance, the up-regulation of *DI* in *gp150* mutant eye discs might be due to impaired N signaling, as N signaling can often cause down-regulation of *DI* expression in signal-receiving cells. Further work is needed to distinguish these possibilities.

N signaling is known to be regulated through mechanisms such as site-specific proteolysis and glycosylation (for review see Artavanis-Tsakonas *et al.*, 1999; Mumm and Kopan, 2000). In addition, N is recognized and ubiquitinated by an E3 ubiquitin ligase Itch (Qiu *et al.*, 2000), suggesting that N stability could be regulated through the proteasome-mediated pathway. An Itch-related *Drosophila* protein, Suppressor of deltex, also negatively regulates N signaling (Cornell *et al.*, 1999). Another negative regulator of N, Numb, has recently been shown to be an endocytic protein (Santolini *et al.*, 2000), suggesting that Numb regulation of N could occur in the endocytic pathway. The regulation of DI is also complex. For instance, DI is proteolytically processed so its extracellular domain can be released (Klueg *et al.*, 1998; Qi *et al.*, 1999). From this work, we propose that Gp150 might function in intracellular vesicles, which include endosomes, to adjust appropriate intracellular levels of the DI protein for modulating N signaling.

As the DER RTK signaling pathway is required for cell specification in the eye, Gp150 might also affect DER-mediated inductive signaling. Our data do not support this model, as mutations in two major components of the DER pathway, Ras1 and MAPK, did not exhibit any apparent dominant effect on the *gp150* mutant eye phenotypes.

Moreover, reduction of *gp150* function did not modify eye phenotypes caused by gain-of-function mutations such as *sev-Ras^{V12}* and *sev-raf^{tor}* (data not shown).

Our functional analysis of Gp150 indicates that both the extracellular and intracellular domains of Gp150 are essential (Dhulkotia *et al.*, 2000 and data not shown). The extracellular domain of Gp150 contains 18 LRR motifs that might provide a scaffold for mediating protein–protein interactions (Kobe and Deisenhofer, 1995). In addition, it has several unique structural features that are conserved during evolution (Dhulkotia *et al.*, 2000). These features include specific potential *N*-glycosylation sites, cysteine motifs and acidic regions that flank the LRR region. Although the intracellular domain is short, it contains three conserved tyrosine phosphorylation motifs (Dhulkotia *et al.*, 2000) that are potential targets of tyrosine kinases (e.g. Dsrc) and receptor tyrosine phosphatases (e.g. DPTP10D; Fashena and Zinn, 1997). The conserved tyrosine phosphorylation motifs might be used for interaction with SH2 domains of some docking proteins. Further studies are required to reveal the functional significance of these conserved structural motifs, which should help reveal mechanisms of Gp150 action in cell fate control and tissue morphogenesis.

Materials and methods

Genetics and histology

l(2)k11107 and *l(2)k11120* (Torok *et al.*, 1993) are referred to as *gp150¹* and *gp150²*, respectively. Based on the mutant eye and bristle phenotypes, these mutations were mapped to the 58D position of the second chromosome. All *gp150* mutations fail to complement *Df(2R)02311* (G.M.Rubin, University of California at Berkeley), a deletion of the 58D1–5 region and several other larger deficiencies (Kerrebrock *et al.*, 1995). On the *l(2)k11107* chromosome, there is another recessive rough eye mutation, which is not associated with *gp150*. We subsequently isolated two EMS alleles, *gp150³* and *gp150⁴*, by a non-complementation assay from a screen of 6167 F₂ lines. Mutant alleles such as *gp150²* and *gp150³* can be considered as genetic nulls. This is based on the fact that there are ~35% (*n* = 522) mutant ommatidia in *gp150²/gp150²* flies while the number is 27% (*n* = 532) in *gp150²/Df(2R)02311*, and there are ~45% (*n* = 484) mutant ommatidia in *gp150²/gp150³* flies while the number is 29% (*n* = 229) in *gp150³/Df(2R)02311*. In a local P transposon-hopping experiment, we isolated nine *gp150* P alleles (P2, P6, P8, P10, P11, P12, P13, P15 and P16) by inducing transposition of *P[lacW]* inserted at 58E1–2 on the *l(2)k08316* chromosome (Torok *et al.*, 1993). The mutant phenotypes can be frequently reverted to wild type by P-element excision in four of the six tested P alleles (P2: 81%, *n* = 94; P6: 0%, *n* = 37; P8: 86%, *n* = 86; P10: 69%, *n* = 42; P15: 39%, *n* = 56; P16: 0%, *n* = 90). P-element transposition was induced by introducing a chromosome carrying a transposase gene (Robertson *et al.*, 1988). *FRT42 gp150²* and *FRT42 gp150³* chromosomes were generated through meiotic recombination. Mutant clones for *gp150[−]* were produced as described (Xu and Rubin, 1993). Other mutations used in this work include *N^{55e11}*, *Df^{RP}*, *Df^{6B}* (a temperature-sensitive allele), *Ras^{1e1B}*, *Ras^{1e2F}*, *rf^{S-135}*, *shi^{ts1}*, *sev-Ras^{V12}* and *sev-raf^{tor}*. The *P[UAS-fng^{ADD-myc}]* line was a gift from M.Freeman, MRC Laboratory of Molecular Biology, Cambridge, UK and the *P[UAS-GFP-DmRab7]* line was a gift from M.Gonzalez-Gaitan, Max Planck Institut für biophysikalische Chemie. Scanning electron microscopy (SEM) and adult eye sections were conducted as described (Lai *et al.*, 1996). Plastic sections were analyzed using a Zeiss Axioplan compound light microscope.

Molecular analysis and germline transformation

Plasmid rescue was performed to isolate the genomic sequence flanking the P insertion site in both P2 and P8 alleles. Three cDNA clones (DDBJ/EMBL/GenBank accession No. AY029705) were isolated after screening 7.2×10^5 recombinants from an eye disc cDNA library (G.M.Rubin). Sequence analyses demonstrated that a deduced full-length protein is identical to Gp150 (Tian and Zinn, 1994). A 16 kb sequence was cloned

from a genomic DNA library (K.Moses, Emory University School of Medicine). The *gp150*-coding region is restricted to a 4 kb sequence containing the third, fourth, fifth and sixth exons. The first (46 bp) and second (72 bp) exons were found 7220 bp upstream of the coding region. To overexpress *gp150* in eye discs, a 4.2 kb full-length *gp150* cDNA was cloned into *pGMR* vector (Hay *et al.*, 1994), which drives gene expression in all cells posterior to the MF. Five independent *P[GMR-gp150]* were produced through germline transformation (Rubin and Spradling, 1982). Standard methods for DNA analysis were used (Sambrook *et al.*, 1989).

Immunocytochemistry and western blot analysis

Mouse polyclonal anti-Gp150 serum was made against the N-terminal region (amino acids 5–192) of Gp150. The serum was diluted 400-fold in a 0.1 M phosphate buffer pH 7.2 with 0.1% Triton X-100 for immunostaining. Third instar eye discs were dissected and fixed in a paraformaldehyde–lysine–phosphate buffer (Tomlinson and Ready, 1987) for 45 min on ice, which was followed by incubation with the anti-Gp150 antibody overnight at 4°C. Texas Red-conjugated anti-mouse IgG was the secondary antibody (Vector Laboratories). For double-labeling experiments, fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG and FITC-conjugated anti-guinea pig IgG secondary antibodies were used (Vector Laboratories). Images were collected on a Bio-Rad MRC-1024 confocal laser scanning system. Other primary antibodies include mouse anti-Boss (used in 1:2000 dilution; S.L.Zipursky, University of California at Los Angeles), mouse anti-Cut (1:10) and mouse anti-Elav (1:400; G.M.Rubin), mouse anti-Hairy (1:1; S.Carroll, University of Wisconsin), rabbit anti-Myc (1:500; Santa Cruz Biotechnology, Inc.), mouse anti-Notch (1:1000; C458.2H, the Developmental Studies Hybridoma Bank at the University of Iowa), guinea pig anti-Delta (1:3000; GP581, M.A.T.Muskavitch, Boston College, MA), rabbit anti-Ato (1:10 000; Y.N.Jan, University of California at San Francisco) and rabbit anti-BarH1 (1:100; K.Saigo, University of Tokyo). Biotinylated secondary antibodies for mouse and rabbit IgG and the Vectastain ABC kit (Vector Laboratories) were used for signal detection. FITC-conjugated phalloidin (1.3 µM; Sigma) was used for detecting actin filaments that outline apical cell surface. The *sh¹⁵¹* mutants were heat treated at 29°C for 7 h before their third instar eye discs were dissected for antibody staining.

For western analysis, the Gp150 antibody (1:1000) was used to probe a blot containing protein extracts of eight pairs of third instar larval eye discs/brain tissues for each lane. To determine whether equal amounts of proteins were loaded in each lane, the same blot was stripped and reprobed for the 70 kDa Fascilin I (Fas I) protein by anti-Fas I antibody (1:3000; mAb6D8; C.Goodman, University of California at Berkeley). A peroxidase-labeled anti-mouse IgG was the secondary antibody (Boehringer Mannheim) and the BM chemiluminescence blotting kit (Boehringer Mannheim) was used for signal detection.

Cell culture and DNA transfection

Drosophila Schneider S2 cells were grown in Schneider's medium supplemented with 10% fetal bovine serum (Gibco-BRL). According to the instructions of the Tfx™-20 reagent (Promega), 10⁶ cells were transfected with 2 µg of each expression construct. Both *pmt-gp150myc* (Fashena and Zinn, 1997) and *pmt-Delta* (Fehon *et al.*, 1990) were induced via addition of 0.7 mM CuSO₄ for 24 h. Cells were fixed and labeled as in Fehon *et al.* (1990). Primary antibodies include anti-Delta antibody (Gp581; 1:3000) and anti-Myc ascites (1:1000; G.M.Rubin).

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