

A Genetic Screen in *Drosophila* for Genes Interacting With *senseless* During Neuronal Development Identifies the Importin *moleskin*

Kathryn L. Pepple,^{*,1} Aimée E. Anderson,^{*,1} Benjamin J. Frankfort^{*,2} and Graeme Mardon^{*,†,‡,§,***,3}

^{*}Department of Molecular and Human Genetics, [†]Department of Pathology, [‡]Department of Neuroscience, [§]Department of Ophthalmology and ^{***}Program in Developmental Biology, Baylor College of Medicine, Houston, Texas 77030

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ABSTRACT

Senseless (*Sens*) is a conserved transcription factor required for normal development of the *Drosophila* peripheral nervous system. In the *Drosophila* retina, *sens* is necessary and sufficient for differentiation of R8 photoreceptors and interommatidial bristles (IOBs). When *Sens* is expressed in undifferentiated cells posterior to the morphogenetic furrow, ectopic IOBs are formed. This phenotype was used to identify new members of the *sens* pathway in a dominant modifier screen. Seven suppressor and three enhancer complementation groups were isolated. Three groups from the screen are the known genes *Delta*, *lilliputian*, and *moleskin/DIM-7 (msk)*, while the remaining seven groups represent novel genes with previously undefined functions in neural development. The nuclear import gene *msk* was identified as a potent suppressor of the ectopic interommatidial bristle phenotype. In addition, *msk* mutant adult eyes are extremely disrupted with defects in multiple cell types. Reminiscent of the *sens* mutant phenotype, *msk* eyes demonstrate reductions in the number of R8 photoreceptors due to an R8 to R2,5 fate switch, providing genetic evidence that Msk is a component of the *sens* pathway. Interestingly, in *msk* tissue, the loss of R8 fate occurs earlier than with *sens* and suggests a previously unidentified stage of R8 development between *atonal* and *sens*.

SENS is a zinc-finger transcription factor that is necessary and sufficient for the development of the founding cell type of the *Drosophila* retina, the R8 photoreceptor (FRANKFORT *et al.* 2001; FRANKFORT and MARDON 2002). It is also necessary for development of the entire *Drosophila* peripheral nervous system (PNS). In the absence of *Sens*, sensory organ precursor (SOP) cells are specified but do not develop, and the corresponding sensory organs fail to form (NOLO *et al.* 2000). In addition, ectopic expression of *Sens* in the thorax generates a massive overproduction of mechanosensory bristles or macrochaete. These data indicate that *sens* acts near the top of the developmental cascade that specifies sensory organ fate. Furthermore, *Sens* is a member of a family of zinc-finger transcription factors termed the Gfi/Pag-3/Sens (GPS) proteins (JAFAR-NEJAD and BELLEN 2004). These conserved proteins are required for normal neural development in many organisms including humans, mice (BELL *et al.* 1995), chicken (FUCHS *et al.* 1997), zebrafish (DUFOURCQ *et al.* 2004), *Caenorhabditis elegans* (JIA *et al.* 1996), and the house fly (KASAI and SCOTT 2001). In mammals, the

Sens homolog Gfi-1 is involved in neurodevelopment of the inner ear hair cells (WALLIS *et al.* 2003) and cerebellar Purkinje cells (TSUDA *et al.* 2005). Mice lacking Gfi-1 are deaf and exhibit ataxia typical of inner ear balance defects (WALLIS *et al.* 2003).

Gfi-1 and its paralog Gfi-1b are also involved in hematopoiesis (ZENG *et al.* 2004; DUAN and HORWITZ 2005) and immune system function (SCHMIDT *et al.* 1998b; KARSUNKY *et al.* 2002; ZHU *et al.* 2002; HOCK *et al.* 2003; YUCCEL *et al.* 2003; ZENG *et al.* 2004; RATHINAM *et al.* 2005) and have been implicated in disease processes including neutropenia (KARSUNKY *et al.* 2002; PERSON *et al.* 2003), carcinogenesis (GILKS *et al.* 1993; SCHMIDT *et al.* 1996, 1998a; HOCK *et al.* 2004; KAZANJIAN *et al.* 2004; SHIN *et al.* 2004; DWIVEDI *et al.* 2005), and neurodegenerative disease (MOROY 2005; TSUDA *et al.* 2005). The expression of both *sens* and *Gfi-1* is positively regulated by the bHLH proneural gene *atonal/Math-5*. In the absence of *atonal* in *Drosophila*, no adult eye is formed due to the failure of R8 photoreceptor specification (JARMAN *et al.* 1994). In mice, loss of *Math-5* results in a severe reduction of *Gfi-1* expression in the retina and loss of most retinal ganglion cells (BROWN *et al.* 2001; WANG *et al.* 2001; YANG *et al.* 2003). The remarkable similarity in function and regulation within the GPS family of proteins suggests that they are part of an evolutionarily conserved neurodevelopment pathway. Surprisingly, in neural development, only the proneural genes have been identified as transcriptional

¹These authors have contributed equally to this work.

²Present address: University of Texas Medical School, Houston, TX 77026.

³Corresponding author: Department of Pathology, Room T222, Baylor College of Medicine, 1 Baylor Plaza, Houston, TX 77030.
E-mail: gmardon@bcm.tmc.edu

targets of Sens. Regulation of proneural transcription by Sens provides amplification of the signal required for selection of the SOP cell (NOLO *et al.* 2001; JAFAR-NEJAD and BELLEN 2004). Due to the importance of Sens in normal development, it is unlikely that proneural genes are its only targets in normal development. Previously, we described a phenotype generated by the expression of *UAS-sens* in undifferentiated cells by the *lozenge-GAL4* driver (referred to as *ls*) that leads to the massive overproduction of interommatidial bristles (IOBs) (FRANKFORT *et al.* 2004). In addition, ectopic Sens is still capable of generating IOBs in the absence of the *ac* and *sc* proneural genes normally required for bristle development. This result supports the hypothesis that the proneural genes are not the only targets of Sens and provides us with a powerful tool for identifying new genes required in this developmental pathway. Identification of other genes acting with Sens in *Drosophila* will lead to a better understanding of normal neural development in the fly and provide insight for potential homologous pathway members in mice and humans.

A genomewide mutagenesis screen is a rapid and powerful method for generating and identifying new genes. The *Drosophila* eye, with its crystalline array of thousands of photoreceptor cells and bristle neurons (CAGAN and READY 1989), is an ideal system for screens that identify genes required for normal neuronal development. In the wild-type eye, each unit eye or ommatidium is a repeating unit with an invariant arrangement of eight photoreceptor cells, termed R1–R8, four lens-secreting cone cells, two primary pigment cells, six secondary pigment cells, and three tertiary pigment cells and three bristle complexes (each composed of four cells) situated at alternating vertices (see Figure 1 for a labeled example). The bristles and the secondary and tertiary pigment cells form a lattice, resulting in the hexagonal shape of each ommatidium and giving the eye a regular, tiled appearance. Perturbations of the normal structure of the eye are readily visible under the light microscope and make the eye particularly useful in genetic screens.

The structure of the adult eye begins to emerge during the early third larval instar. At the posterior of the field of undifferentiated epithelial cells of the eye imaginal disc, a visible indentation termed the morphogenetic furrow is formed. Within and immediately ahead of the furrow, changes in gene expression required for the initiation of photoreceptor development occur (PAPPU and MARDON 2004). The morphogenetic furrow progresses toward the anterior of the disc, leaving clusters of newly differentiating photoreceptor cells in its wake. At the molecular level, the proneural gene *atonal* (*ato*) is expressed in a stripe immediately anterior to the furrow and gradually resolves into regularly spaced groups of cells, the proneural clusters (PNCs). This expression is eventually resolved into single cells, corresponding to the nascent R8s (JARMAN

et al. 1994). At this stage, Sens expression is dependent on *ato* (NOLO *et al.* 2000; FRANKFORT *et al.* 2001) and strongly coincides with Ato at the three-cell stage (the R8 equivalence group) and persists beyond Ato expression in single R8 cells posterior to the furrow. *sens* in turn is necessary and sufficient for differentiation of the R8 cell (FRANKFORT *et al.* 2001). Later in eye development, *sens* is required for interommatidial bristle development (FRANKFORT *et al.* 2004). Expression of *sens* in this process is dependent on a different set of proneural genes, the genes of the *achaete-scute* complex. The interommatidial bristle is composed of four cells (the socket, shaft, glia, and neuron) that are clonally derived from a single cell that begins to express Sens 3–6 hr after puparium formation (APF) (CAGAN and READY 1989; FRANKFORT *et al.* 2004). By 24 hr APF all four cells of the bristle complex have been formed and express Sens, while at 48 hr APF Sens is no longer expressed in the bristle complex, but is still expressed in the developing R8 photoreceptor.

Here we describe a forward genetic screen using an ectopic Sens genotype (*lz-GAL4 UAS-sens*, termed “*ls*”) in which the ommatidia of the adult eye have a rough, irregular appearance and numerous extra IOBs. We crossed *ls* flies to ethyl methanesulfonate (EMS)-mutagenized *w⁻* flies and screened 1.1×10^5 progeny for enhancement or suppression of the rough eye and extra bristle phenotype. We isolated and mapped seven suppressor and three enhancer complementation groups. Three complementation groups were identified as the known genes *lilliputian* (*lilli*), *Delta* (*Dl*), and *moleskin/dim-7* (*msk*). We discuss the relationship of these genes to the ectopic Sens phenotype, as well as the relationship between these genes and *sens* in normal development. Finally, we show that Msk, a homolog of the nuclear import factor Importin 7, is necessary for normal eye development and acts in R8 development at a previously unidentified stage upstream of Sens.

MATERIALS AND METHODS

Mutagenesis: Isogenized *w¹¹¹⁸* males were collected and aged for 3 days. On day 3, males were starved for 8 hr and then added to bottles containing filter paper soaked with 25 mM EMS (Sigma, St. Louis) and 1% sucrose in water. Males were exposed to the mutagen for 12–15 hr and then allowed to recover on fresh media for 24 hr before mating to *lz-GAL4, UAS-sens/FM7* females. Non-FM7 F₁ progeny were scored for suppression or enhancement. Modified F₁ progeny were crossed to *lz-GAL4, UAS-sens/FM7* females or *lz-GAL4, UAS-sens/Y* males, and F₂ male progeny that showed the same modification were then backcrossed to the *lz-GAL4, UAS-sens/FM7* females for at least three generations to separate second-hit lethal mutations and weak modifiers from strong single-hit modifiers. Modification of *ls* caused by X chromosome mutations was scored in F₁ females. In these flies, the mutation was located in *trans* to the *ls* chromosome. Due to the high rate of recombination that separates *lz-GAL4* from *UAS-sens*, modified F₁ females were always backcrossed to *ls* males to ensure that

modification could be scored in the F₂. In the F₂ only modified males were selected. Then, in the F₃, only *ls* females balanced by *FM7* were scored and collected. Thus, a modifying mutation located on the X could be recovered only when a recombination event had occurred in the F₁ female between the *ls* chromosome and the mutant chromosome. This recombination event happened either very rarely or not at all, because we did not recover any X chromosome mutants. After multiple backcrosses through females, F₅ modified females were crossed to *w*; *Bc,Elp/CyO* and *w*; *TM3/TM6B* males. In the F₆ single *ls* males that carried a balancer (*CyO* or *TM3*) and showed modification were crossed first to *w*⁻ virgins and then to virgins of the corresponding balancer stock (*w*; *Bc,Elp/CyO* or *w*; *TM3/TM6B*). The female progeny of the *w*⁻ cross were scored to determine segregation. Once the chromosome was determined, the progeny from the proper balancer stock cross were collected to generate the final balanced lines. Balanced mutations were then tested for failure to complement a set of Bloomington deficiencies that were able to modify *ls*. Complementation analysis was performed among all mutants on each chromosome.

Fly stocks and genetics: Fly stocks used included *w*¹¹¹⁸, *Dl^{pp}*, *Dl^{h2}*, *lilly⁰⁰⁶³²*, *msk²*, *msk⁴*, *lz-GAL4,UAS-sens/FM7 (ls)*, *w;ey-GAL4,UAS-flp/CyO,hs-hid*; *cl,GMR-hid,FRT79D/TM6B (EGUF79D)*, *lz-GAL4/FM7; UAS-ro/CyO*, *sc^{10.1},lz-GAL4 (FRANKFORT et al. 2004)*, *UAS-msk* (gift from L. Perkins), *UAS-Sens* (on the second chromosome), *sca-lacZ*, *RM104*, *BBO2*, *UAS-GFP*, *UAS-Dl*, *w; C96-GAL4,UAS-hrs/TM6B* (gift from H. Bellen), and, from the Bloomington stock center, the first, second, and third chromosome deficiency kits. The molecularly mapped *P* elements used to fine map *S(ls) 1*, *S(ls) 3*, *S(ls) 5*, *S(ls) 7*, and *E(ls) 3* were obtained from the P-screen database (BELLEN et al. 2004). Molecular information is available at <http://flypush.imgen.bcm.tmc.edu/pscreen/>. *P* elements flanking each group are as follows: *S(ls) 1*, KG03264 and KG04159; *S(ls) 3*, KG00038 and KG05698; *E(ls) 3* and *S(ls) 7*, EY07182 and KG03062; and *S(ls) 5*, KG05323 and KG00569.

Generation of mosaic retina and whole mutant eyes: The *msk²* allele was recombined onto a third chromosome containing an *FRT* element at either 79D or 80B. To generate pupal retinas mosaic for *msk²*, males of the genotype *ls; msk²,FRT80B/TM6B* were crossed with *hsflp; ubi-GFP,FRT80B* virgins. Progeny of this cross were heat-shocked for 1 hr at 37° 36–38 hr after egg laying. White prepupae were collected (0 hr APF) and aged at 18° for 96 hr (equivalent to 48 hr at 25°). To generate whole eyes mutant for *msk*, the EGUF system was utilized (STOWERS and SCHWARZ 1999). Males of the genotype *w; ey-GAL4,UAS-flp/CyO,hs-hid; cl,GMR-hid,FRT79D/TM6B* were crossed to *w; msk², FRT79D/TM6B* or *w; FRT79D* for control animals. *w; msk², RM104, FRT79D/TM6B* or *w; RM104, FRT79D/TM6B* females were used for the experiments in Figure 10, A–H. To generate *msk* whole mutant eyes carrying the *BBO2* or *sca-lacZ* reporters, females of the genotype *w; reporter/CyO,hs-hid; msk²(or +) FRT79D/TM6B* were used. Progeny were heat-shocked 72 hr after egg laying (AEL) for 1 hr at 37°. At 120–144 hr AEL non-tubby wandering third instar larvae were dissected for larval immunohistochemistry. Adults were collected for sectioning and scanning electron microscopy (SEM) analysis as described below.

Antibody staining: The following primary antibodies were used: rabbit anti-GFP (1:1000; Molecular Probes, Eugene, OR), guinea-pig anti-Sens (1:800, a gift from Hugo Bellen), rabbit anti-Atonal (1:5000, gift of Andrew Jarman), rabbit anti-β galactosidase (1:800, MP Biomedicals Cappel), rat anti-Elav (1:500, Developmental Studies Hybridoma Bank), mouse anti-Cut (1:100, Developmental Studies Hybridoma Bank), and mouse anti-Arm (1:500, Developmental Studies Hybridoma Bank). Goat anti-rat Cy3 and goat anti-rabbit Cy3 secondary

antibodies were obtained from Jackson Laboratories (West Grove, PA). Goat anti-mouse Alexa, goat anti-guinea pig Alexa, and goat anti-rabbit Alexa secondary antibodies were obtained from Molecular Probes. All secondary antibodies were used at a 1:500 dilution.

For larval time points, eye imaginal discs were dissected from wandering third instar larvae into 1× PBS (0.1 M phosphate, pH 7.2, 150 mM NaCl) on ice. Discs were then fixed in 4% formaldehyde in 1× PBS for 25 min on ice. For Boss staining, discs were fixed in 2% paraformaldehyde, 10 mM NaIO₄, 75 mM lysine, 3.5 mM NaPO₄, pH 7.2 (PLP) for 40 min on ice. Discs were then washed for 10 min each in 1× PBS; in PBS with 1% BSA, 0.3% Triton X-100, and 0.3% sodium deoxycholate (PAXD); and in PAXD with 5% heat-inactivated and filtered normal goat serum (PAXDG) and then incubated with primary antibody diluted in PAXDG overnight at 4°. Discs were washed three times in PAXDG followed by incubation with secondary antibody for 2 hr at room temperature. Discs were then washed for 10 min each in PAXDG, PAXD, and PBS followed by fixation in 4% formaldehyde in PBS for 15 min at room temperature. Discs were washed one final time for 10 min in PBS and then equilibrated in Vectashield overnight at 4°. Fluorescent images were captured with a Zeiss LSM 510 confocal microscope. Images were processed using Image J and Adobe Photoshop software.

SEM: Adult animals were collected and aged on fresh food for 2–3 days and then taken through an EtOH dehydration series. The flies were washed in 1 ml of 30, 50, 70, 90, 100, and 100% EtOH in water for 10–12 hr per step. Hexamethyldisilazane (HMDS; Electron Microscopy Sciences, Fort Washington, PA) was then used to chemically dry the samples. The flies were washed for 30 min in 500 μl of the following solutions: 75% EtOH + 25% HMDS, 50% EtOH + 50% HMDS, 25% EtOH + 75% HMDS, 100% HMDS, 100% HMDS, and 100% HMDS. The final 100% HMDS wash was allowed to evaporate under vacuum in the presence of anhydrous calcium sulfate (Drierite; W. A. Hammond Drierite, Xenia, OH). The samples were then mounted on carbon-conductive tabs (Ted Pella, Redding, CA) on top of aluminum stubs (Electron Microscopy Sciences) and coated to 40 nm thickness with sputtered platinum. Images were captured with a JSM-5900 scanning electron microscope (Jeol, Tokyo) and processed with Adobe Photoshop software. The specific alleles pictured in Figure 5 are as follows: (Figure 5, B and F) EMF13, (Figure 5, C and G) EMC7, (Figure 5, D and H) SMB23, (Figure 5, I and M) SFB22, (Figure 5, J and N) SFF117, (Figure 5, K and O) SMD20, and (Figure 5, L and P) SFJ10.

Light microscopy: Adult wings were mounted on a 25-μl drop of xylene and then covered with Cytoseal XYL (Richard-Allan Scientific, Kalamazoo, MI) and a glass coverslip. Adult eye sections were prepared and sectioned as described in TOMLINSON and READY (1987). Images were captured using a Zeiss Axioplan2 microscope and a Zeiss Axiocam digital camera. Images were processed with Adobe Photoshop software.

Cell counting and intensity measurement: Projections of confocal microscope stacks were made with ImageJ software (ABRAMOFF et al. 2004). The area, in square micrometers, of twinspace (*msk* +/+) and heterozygous (*msk* -/+) tissue was measured with ImageJ and the number of Sens-positive cells in each area was counted using the ImageJ point selection tool. Data were imported into Microsoft Excel, which was used for all subsequent analysis. The number of cells counted was divided by the area of tissue to give the number of cells per square micrometer. Student's *t*-test was used to test for a significant difference in cell density between the twinspace and heterozygous tissue. To illustrate differences in pixel intensity graphically, the heat-mapping function of Image J was

utilized. Images were then processed with Adobe Photoshop software as described above.

RESULTS

Ectopic expression of Sens in undifferentiated cells generates extra IOBs: Misexpression of *UAS-sens* in the eye under the control of *lozenge-GAL4* (*ls*) generates a roughened eye with many ectopic IOBs (see Figure 1, D and E). Expression of *lozenge-GAL4* begins in undifferentiated cells posterior to the morphogenetic furrow (CREW *et al.* 1997). Later expression is found in the R1, -6, and -7 photoreceptor cells and in cone and pigment cells. Since Sens is sufficient for the R8 and IOB cell fate, we predicted that the *ls* phenotype would likely result from changes in normal ommatidial cell fate caused by ectopic Sens. To determine which cells are affected, we sectioned adult eyes and performed antibody staining

on larval and pupal eye tissue with cell type-specific markers. Interestingly, no photoreceptor cell types are lost, and no ectopic R8 photoreceptor cells are formed despite the presence of ectopic Sens in the third larval instar disc (data not shown and compare Figure 1C to 1F). Therefore, the *ls* genotype is not affecting early cell fate decisions. The ectopic bristles caused by *ls* could be due to recruitment of undifferentiated cells to the bristle fate or a switch in cell fate of an accessory cell type to a bristle cell. Armadillo staining of *ls* pupal discs at 48 hr APF (Figure 1H) demonstrates that in most ommatidia all accessory cells are present, although secondary and tertiary pigment cell are slightly misshapen compared to wild type (Figure 1G; see inset in Figure 1G for cell-type identification). Occasional secondary pigment cells are absent, leading to ommatidial fusions (arrows in Figure 1, F and H). These missing secondary pigment cells may be recruited to the bristle fate, but their numbers cannot account for the massive number of extra IOBs. Therefore, most of the extra IOBs in the *ls* eye are likely recruited from undifferentiated cells that are usually removed by apoptosis during pupal development (BRACHMANN and CAGAN 2003). To provide a comparison of Sens expression in the wild-type and *ls* eye, pupal discs were stained at 48 hr APF. In the wild-type eye, there is one Sens-positive cell in each ommatidium, corresponding to the R8 (Figure 1I). In contrast, the *ls* eye has many Sens-positive cells per ommatidium (Figure 1J).

Delta enhances the *ls* phenotype: Ectopic expression of Sens by *lz-GAL4* recruits many undifferentiated cells to the bristle fate. The increased number of neurons is similar to the neurogenic phenotype seen when Notch

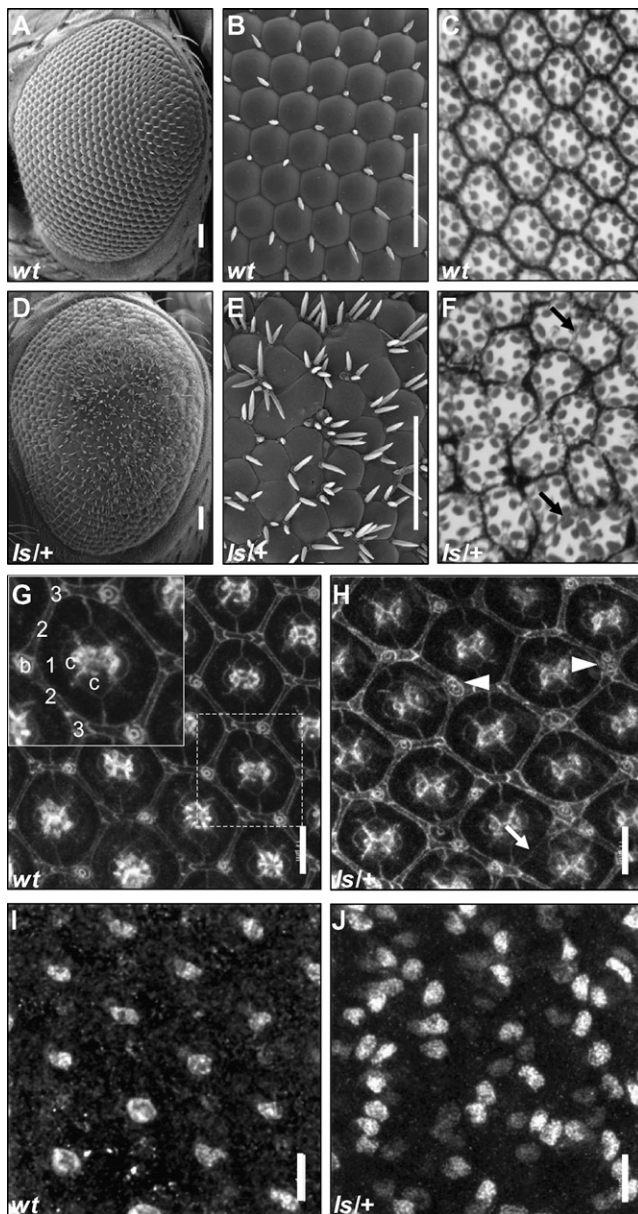


FIGURE 1.—Ectopic Sens recruits cells to the interommatidial bristle fate. The wild-type adult *Drosophila* eye is composed of ~800 ommatidia arranged in a crystalline-like lattice (A–C, G, and I). Each wild-type ommatidium has an interommatidial bristle (IOB) located at every other vertex. Scanning electron micrographs of adult *Drosophila* eyes 200 \times (A and D) or 1000 \times magnification (B and E) are shown. Bars in A–E, 50 μ m. When *UAS-sens* is ectopically expressed using the *lozenge-GAL4* driver (*ls*), extra IOBs are formed and the eye is roughened (D and E); note multiple bristles at a single vertex as well as ectopic bristles outside of the vertices. Thin plastic sections of adult wild-type eyes show the trapezoidal arrangement of normal photoreceptors (C). The normal number of photoreceptors is found in the *ls* eye (F); in contrast, normal morphology is disrupted by misrotation and ommatidial fusions (arrows in F and H) due to pigment cell loss. Staining of wild-type and *ls* 48-hr APF disc cell membranes with Armadillo (Arm) antibody shows the structure of the developing accessory cells (G and H). In the magnified image in G, each ommatidium has four cone cells (c) surrounded by two crescent-shaped primary pigment cells (1), four secondary pigment cells (2), three tertiary pigment cells (3), and three bristles (b). Cells on half the ommatidium are not labeled to aid visualization. Each ommatidium in G has a Sens-positive R8 cell shown in I. In the *ls* eye (H), some ommatidia show multiple bristles (arrowheads) and ommatidial fusions (arrow), as well as multiple Sens-positive cells (J). Bars in G–J, 11 μ m.

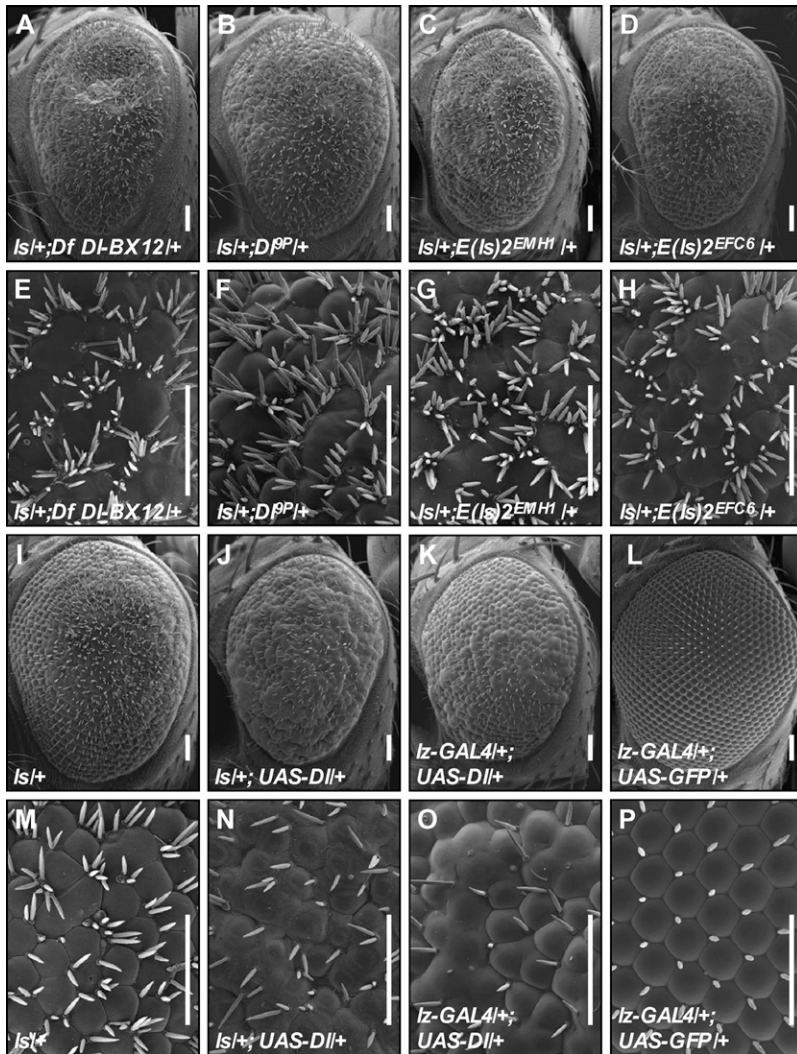


FIGURE 2.—*Delta* modifies *ls* in a predicted manner. *Df(3R) DL-BX12* uncovers *Delta* and was identified as an enhancer of the *ls* phenotype (A and E). A previously identified mutation in *Dl* (*Dl^{pp}*) fails to complement the deficiency (data not shown) and also enhances the *ls* phenotype (B and F). Two EMS mutants were identified as alleles of *Delta*; these also enhance the *ls* phenotype (compare C and G and D and H to I and M). Correspondingly, overexpression of *Delta* (J and N) decreases the number of ectopic bristles produced by *ls* (compare J and N with I and M). Control animals are *lz-GAL4/+; UAS-Dl/+* (K and O) and *lz-GAL4/+; UAS-GFP/+* (L and P).

signaling is decreased. The Notch signaling pathway is responsible for limiting the number of cells in the PNC that achieve the neuronal fate (BRAY 1998; ARTAVANIS-TSAKONAS *et al.* 1999; BAKER 2000; SCHWEISGUTH 2004). When Notch signaling is lost, proneural gene expression fails to resolve into a single cell, thus allowing all cells of the PNC to become a neuron (HARTENSTEIN and POSAKONY 1990; SKEATH and CARROLL 1992; RUIZ-GOMEZ and GHYSEN 1993). Resolution of the PNC into a single SOP results from transcriptional repression of the proneural genes and their targets by the Enhancer of Split [E(spl)] proteins (PAROUSH *et al.* 1994; JIMENEZ and ISH-HOROWICZ 1997; CULI and MODOLELL 1998; GIAGTZOGLU *et al.* 2003). In addition, the ability of Sens to promote the neural fate is decreased when bound by E(spl)m8 (JAFAR-NEJAD *et al.* 2003). These known connections between lateral inhibition and *sens* led us to hypothesize that members of the Notch pathway should modify *ls*. Identification of modifying candidate genes in the Notch pathway would support the idea that genes interacting with *sens* could be identified in an EMS screen for modification of *ls*.

Multiple available alleles of Notch pathway members were crossed with *ls* virgins and F₁ progeny were scored for enhancement or suppression of the *ls* phenotype. Loss of one copy of *Dl* causes strong enhancement of the number of ectopic bristles formed in *ls* and a marked increase in the disorganization of ommatidial architecture (see Figure 2). The enhancement is consistent for two amorphic alleles tested (*Dl^{pp}* and *Dl^{B2}*) (see Figure 2, B and F, and data not shown). Consistent with the candidate gene screen that identified *Dl* as a modifier, in a test screen with the Bloomington deficiency kit, to be discussed later, *Df(3R)DL-BX12* was also identified as an enhancer of *ls* (see Figure 2, A and E). This deficiency uncovers the *Delta* locus. We were initially surprised that we did not see enhancement with other components of the Notch signaling pathway. However, since only dominant interactions were tested, this indicates only that the *ls* phenotype is more sensitive to loss of one copy of *Delta* than any other pathway member.

Decreasing *Dl* in the presence of ectopic Sens leads to an increased number of IOBs. Therefore, we hypothesized that increasing *Dl* in the *ls* background should

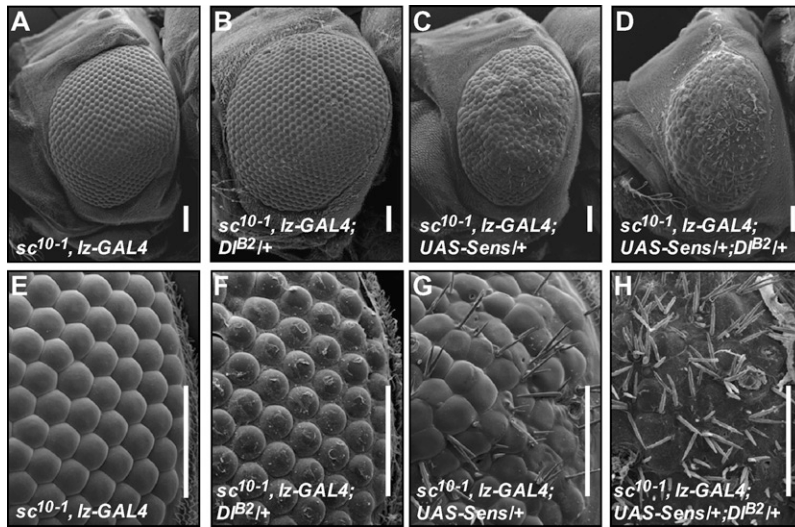


FIGURE 3.—*Delta* modulates *sens* function in the absence of proneural genes. The *sc*¹⁰⁻¹ deficiency removes the *ac/sc* locus, and hemizygous males lack IOBs (A and E). Loss of one copy of *Dl* does not affect this phenotype (B and F). In the *sc*¹⁰⁻¹ background, ectopic *Sens* leads to the formation of IOBs and a roughening of the eye surface (C and G). Removal of one copy of *Dl* increases the number of bristles that are formed and leads to a further loss of normal ommatidial surface architecture (D and H). Images in A–D are shown at 200× magnification, and images in E–H are shown at 1000×. Bars, 50 μm.

decrease the number of IOBs formed. This was tested by expressing *UAS-Dl* with the *lz-GAL4* driver along with *UAS-sens*. As predicted, in the *ls/+; UAS-Dl/+* eye (Figure 2, J and N), a decreased number of bristles is formed compared to the *ls* eye (Figure 2, I and M). Together, these data demonstrate that the *ls* phenotype is modified in a manner predicted by known interactions between lateral inhibition and *Sens*-dependent neural development.

One possibility that these data do not address is that the effect of *Dl* on the *ls* phenotype could be mediated through the proneural genes and not by a direct effect on *Sens*. *sens* expression is downstream of proneural gene expression, and during SOP formation proneural gene expression is refined by lateral inhibition. With decreased expression of *Delta*, the resolution of the proneural field is impaired, and more than one SOP can be selected (PARKS and MUSKAVITCH 1993). This is due to the derepression of the proneural genes with decreased lateral inhibition (PARKS *et al.* 1997). It is possible that the extra SOP formation that results from derepressed proneural expression explains the increased number of bristles seen in the *Dl*-enhanced *ls* eye. To determine if the enhancement of *ls* is dependent on the effect of *Dl* on proneural gene expression, we tested the ability of *Dl* to enhance *ls* in the absence of the relevant proneural genes. The *sc*¹⁰⁻¹ deficiency removes both *ac* and *sc*, the proneural genes required for bristle development. Hemizygous males of this genotype can be collected as pharate pupae and completely lack IOBs (see Figure 3, A and E). With the addition of the *ls* chromosome, bristles can once again be formed even in the absence of *ac* and *sc* (Figure 3, C and G). As we have reported previously, the ability of ectopic *Sens* to form bristles in the absence of the *ac/sc* locus has regional dependence. The posterior region of the eye shows much stronger induction of bristles than the anterior of the eye (FRANKFORT *et al.* 2004). This is likely due to the progressive nature of the morphogenetic furrow that leads to a longer exposure of cells in the posterior region of the eye to

ectopic *Sens*. When a single copy of *Delta* is removed in an *ls* fly that lacks *ac* and *sc*, the number of bristles formed is increased (Figure 3, D and H). Thus, we see an enhancement of the *ls* phenotype by *Delta* even in the absence of the bristle proneural genes. Anterior, middle, and posterior regions of the eye show enhancement in the presence of *Dl* consistent with the graded response to *Sens*. These data show that the enhancement of *Dl* on the *ls* phenotype is not dependent on the refinement of proneural gene expression and support previous evidence that lateral inhibition downregulates *Sens* function. This is consistent with a body of work that describes the effect of lateral inhibition not only at the level of proneural gene expression, but also at the level of regulation of proneural target gene expression and function (CULI and MODOLELL 1998; GIAGTZOGLU *et al.* 2003; JAFAR-NEJAD and BELLEN 2004).

Taken together, these data show that we have created an ectopic *Sens* phenotype that is dominantly modifiable in a predictable way by *Dl*, a key gene in a pathway known to interact with *Sens* in neural development. This suggested that a genomewide mutagenesis screen would identify new genes interacting with *senseless* by their ability to modify *ls*. The utility of the screen was confirmed as two new alleles of *Delta* were identified during the screen as enhancers of *ls* (Figure 2, C, D, G, and H).

An F₁ dominant modifier screen identifies 10 complementation groups interacting with *Sens*: An F₁ dominant modifier EMS mutagenesis screen was performed in which 110,000 animals were screened. *w*¹¹¹⁸ males were mutagenized and crossed to *ls/FM7* virgins (Figure 4). F₁ progeny were scored for suppression or enhancement of the *ls* phenotype. One thousand one hundred thirty-five suppressors and 501 enhancers were identified; these F₁ animals were backcrossed to *ls* flies. After at least three generations of backcrosses of mutant females to *ls* males, 117 suppressors and 11 enhancers were isolated and mapped to the second or third

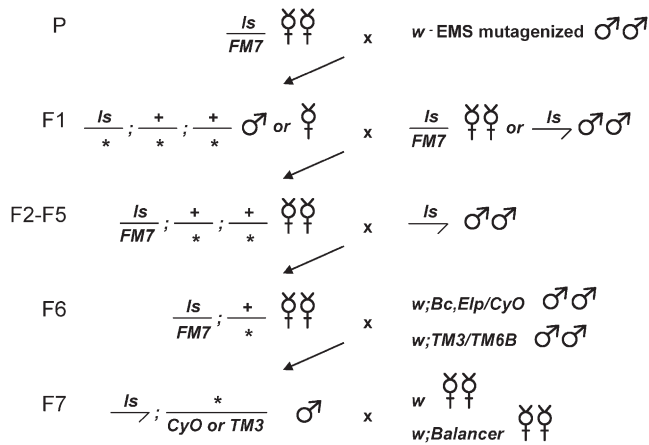


FIGURE 4.—Crossing scheme for the F₁ dominant modifier screen. Isogenized *w*¹¹⁸ males were mutagenized with 25 mM EMS and crossed to females with the *lz-GAL4,UAS-sens* chromosome (*Is*). F₁ progeny that showed enhancement or suppression of the *Is* phenotype were backcrossed to *Is* males or females. From F₁ male backcrosses, F₂ female progeny balanced by *FM7* and carrying a modifying mutation were backcrossed to *Is* males for at least three generations. From F₁ female backcrosses, F₂ *Is* males carrying a modifying mutation were collected and crossed to *Is/FM7* virgins. The F₃ balanced female progeny carrying a modifying mutation were backcrossed to *Is* males for at least three generations. F₆ *Is/FM7* females still carrying a modifier were crossed in two separate batches to second and third chromosome balancer males. Single F₇ *Is* males with a modifier and a balancer chromosome were crossed in series to *w* virgins and then to virgins from the corresponding balancer stock to map the mutation to a chromosome and produce a balanced stock.

chromosome (see Table 1). Once mutations were mapped to a chromosome, balanced stocks were generated. Thirty-two mutations mapped to the second chromosome while 96 mapped to the third. We did not identify any mutations on the X chromosome, in large part due to the location of the *lz-GAL4* and *UAS-sens* *P*-element insertions on the X chromosome and our crossing scheme for recovering mutations (see MATERIALS AND METHODS). This outcome was not considered a problem for the success of the screen since no X chromosome deficiency was identified as a modifier of *Is*, suggesting that few loci on the X would be dominant modifiers. Seventy-nine mutants were either lethal mutations or segregated with a second lethal hit as judged by their inability to homozygose, while 49 mutant chromosomes were homozygous viable. Thirty-six lethal mutations were assigned to 10 complementation groups on either the second or the third chromosome, while 43 lethal mutants were single hits. Only 1 of the 49 viable mutants was assigned to a complementation group; the other 48 are either single hits or multiple hits in genes with no readily detectable homozygous phenotype. Examples of modification of the *Is* phenotype by a representative member of each complementation group are shown in Figures 2, 5, and 7 and supplemental Figure 1 at <http://www.genetics.org/supplemental/>.

TABLE 1
Screen summary

	No. of flies	% recovery	
F ₁ flies screened	110,000		
F ₁ modified flies selected	1,636	1.5	
Final balanced stocks	128	0.1	
Complementation group name	Location	No. of alleles	Gene name
<i>S(Is)1</i>	Centromeric 3	4	
<i>S(Is)2</i>	66B6	15	<i>moleskin/dim-7</i>
<i>S(Is)3</i>	88E–88F	3	
<i>S(Is)4</i>	23C1–3	2	<i>lilliputian</i>
<i>S(Is)5</i>	21A	2	
<i>S(Is)6</i>	35E5–6	1	
<i>S(Is)7</i>	89B3–89B4	2	
<i>E(Is)1</i>	42A1–42E6	1	
<i>E(Is)2</i>	92A1–2	2	<i>Delta</i>
<i>E(Is)3</i>	89B3–89B4	4	
Single alleles		92	

Mapping of the complementation groups utilized multiple strategies. Before the EMS screen was initiated, we performed a genomewide deficiency screen using the Bloomington deficiency kit to determine how many and which regions of the genome could modify the *Is* phenotype. At the time this screen was performed, the Bloomington kit contained 210 stocks that uncovered ~70–80% of total euchromatin (Bloomington Stock Center 10/04/02). In this screen, *Is/FM7* virgins were crossed to balanced deficiencies on the second, third, and fourth chromosomes while X chromosome deficiency virgins were crossed to *Is* males. This screen identified 12 deficiencies that modified the *Is* phenotype (see Table 2). For each complementation group from the EMS screen, a rough mapping position was determined either by failure to complement 1 of the 12 modifying deficiencies or by low-resolution meiotic mapping with a kit of molecularly mapped *P{w+}* elements (ZHAI *et al.* 2003). Groups that failed to complement a deficiency were then tested against lethal genes located within the breakpoints of that deficiency. In this way, members of *S(Is)2* were identified as alleles of *moleskin/dim-7*, *S(Is)4* as alleles of *lilliputian*, and *E(Is)2* as alleles of *Delta*. For groups that failed to complement a deficiency, but not a single gene within that deficiency, a round of rough meiotic mapping using five *P{w+}* elements spaced over the region of the deficiency was performed. Alternatively, for groups that did not map to a previously defined modifying deficiency, a rough map position was determined using the *P{w+}* elements in the kit outlined in ZHAI *et al.* (2003), which are spaced every 1–3 Mb. Each complementation group was mapped to all the *P{w+}* elements for the appropriate chromosome and then subjected to a round of intermediate

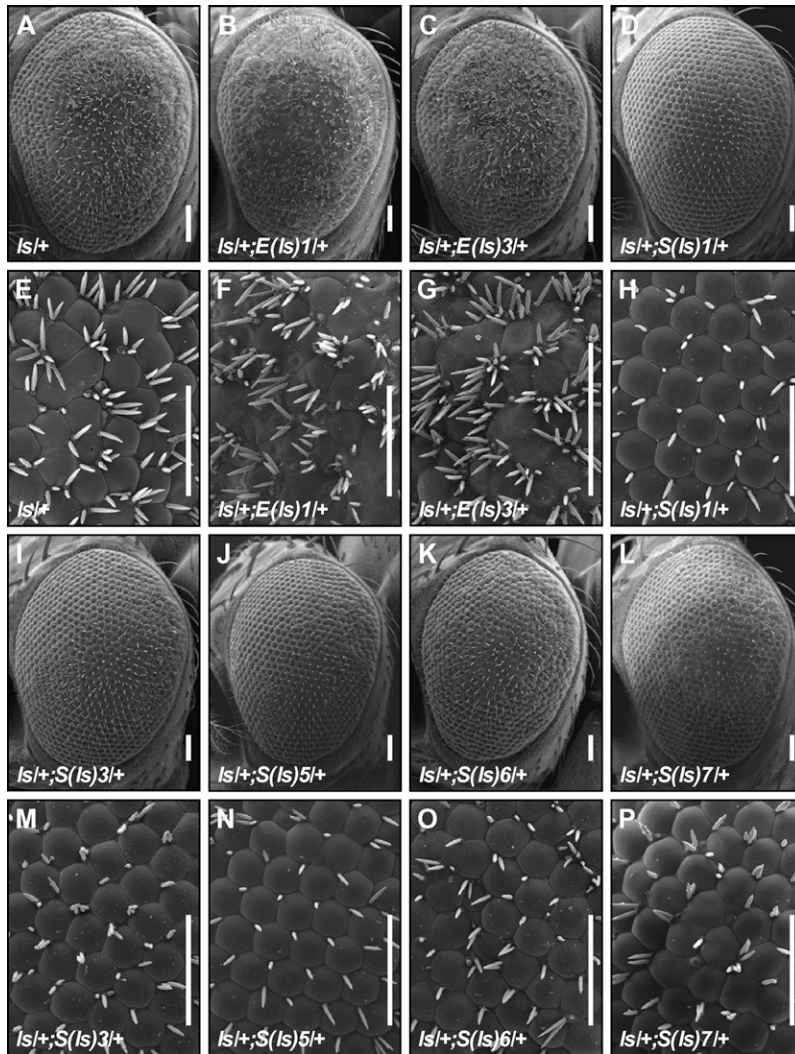


FIGURE 5.—Dominant modification of *ls* by EMS alleles. Thirty-six independent mutations modify the *ls* phenotype (A and E) and form 10 complementation groups. The complementation group name is indicated. Complementation groups representing known genes are not shown in this figure (see Figures 2 and 7 and supplemental Figure 1 at <http://www.genetics.org/supplemental/>). B, C, F, and G show dominant enhancement. More bristles are formed and the external architecture of the eye is more disrupted. D and H–P show dominant suppression of *ls*. The number of bristles formed is decreased and the ommatidial architecture is less disrupted than in *ls*. Bars, 50 μ m.

mapping using *P{w+}* elements spaced at \sim 200-kb intervals between the flanking *P{w+}* elements. This round of mapping was followed by a final round of high-resolution *P{w+}* mapping as described in ZHAI *et al.* (2003). This provided a predicted location within 50 kb. A summary of complementation group location by cytological position in the genome is presented in Figure 6.

***lilliputian* is a suppressor of *ls*, but does not interact specifically with *sens*:** From the Bloomington deficiency kit screen, *Df(2L)C144* was identified as a suppressor of *ls*. *lilliputian* is a gene within *Df(2L)C144* that has been identified as a dominant suppressor of ectopic phenotypes in at least 10 screens (DICKSON *et al.* 1996; PERRIMON *et al.* 1996; NEUFELD *et al.* 1998; GREAVES *et al.* 1999; LI *et al.* 2000; REBAY *et al.* 2000; SU *et al.* 2001; LI and LI 2003; LUSCHNIG *et al.* 2004; ANDERSON *et al.* 2005). Therefore, we tested *lilli* for its ability to modify the *ls* phenotype. *lilli*⁰⁰⁶³² showed strong dominant suppression and failed to complement *Df(2L)C144*. In addition, two independent suppressors of *ls*, corresponding to complementation group *S(ls)4* from the EMS screen, were identified as

alleles of *lilli* by failure to complement *Df(2L)C144* and *lilli*⁰⁰⁶³². *lilli* is an AF4/FMR2 family transcription factor that is required for normal embryogenesis (WITTEW *et al.* 2001). Normal eye cell fate specification is not disrupted in *lilli* mutant clones, but the photoreceptor rhabdomeres are smaller in size than wild-type rhabdomeres (TANG *et al.* 2001; WITTEW *et al.* 2001).

To eliminate genes that modify *ls* due to nonspecific effects on the *GAL4/UAS* system or expression from the *lozenge* locus, all complementation groups were tested in two secondary screens. The first secondary screen tested the ability of mutants to modify a rough-eye phenotype generated by the ectopic expression of *UAS-rough* with the *lz-GAL4* driver (*lz-ro*). Mutations that modify this phenotype as well as the *ls* phenotype could represent genes that are not specific to *sens* interaction, but rather could be affecting transcription from the *lozenge* (*lz*) locus. *lz-Gal4* was generated by a *P{GawB}* insertion in the *lz* locus, so transcription of *GAL4* in these flies is regulated by the factors that normally govern *lz* expression. All mutants and modifying deficiencies were tested against *lz-ro*. The *lilliputian* complementation group

TABLE 2
Deficiencies that modify *ls*

Bloomington deficiency no.	Symbol	Effect	Break points	Single genes identified
90	<i>Df(2L)C144</i>	Su	22F3–4; 23C3–5	<i>lilliputian/S(ls)4</i>
1491	<i>Df(2L)r10</i>	Su	35D1; 36A6–7	<i>S(ls)6</i>
749	<i>In(2R)bw^{VD=2L}Cy^R</i>	Su	42–43; 42A2–3	
3368	<i>Df(2R)cn9</i>	Su	42E; 44C	
3909	<i>Df(2R)59AD</i>	Su	59A1–3; 59D1–4	
1420	<i>Df(3L)pbl-X1</i>	Su	65F3; 66B10	<i>moleskin/S(ls)2</i>
3640	<i>Df(3L)brm11</i>	Su	71F1–4; 72D1–10	
2998	<i>Df(3L)81k19</i>	Su	73A3; 74F	
823	<i>Df(3R)D605</i>	Su	97E3; 98A5	
1007	<i>Df(2R)map9</i>	En	42A1–2; 42E6–F1	<i>E(ls)1</i>
1931	<i>Df(3R)by416</i>	En	85D8–12; 85E7–F1	
3012	<i>Df(3R)Dl-BX12</i>	En	91F1–2; 92D3–6	<i>Delta/E(ls)2</i>

Su, suppression; En, enhancement.

S(ls)4 comprised the only alleles that suppressed the *lz-ro* phenotype. Aside from effects at the *lz* locus, *lilli* could also be affecting some general aspect of transcription in the GAL4 system. To test for this possibility, another test was performed by crossing *lilli* to flies carrying the *C96-GAL4* and *UAS-hrs* transgenes. This genotype leads to the formation of multiple notches along the wing margin, but has no known relationship to *lz* function or expression (see supplemental Figure II at <http://www.genetics.org/supplemental/>). All alleles of *lilli* tested, including *Df(2L)C144* and the screen-generated mutants, strongly suppress the wing notching normally seen with *C96-GAL4*, *UAS-hrs* (supplemental Figure IJ at <http://www.genetics.org/supplemental/>). This strongly suggests that *lilli* suppresses all three gain-of-function phenotypes by suppressing the GAL4/UAS system. This conclusion is consistent with previous studies showing

that *lilli* affects transcription from constructs utilizing *hsp70* promoters for ectopic gene expression (TANG *et al.* 2001; WITTEWERT *et al.* 2001). The *P{GAWB}* element used to generate *lz-GAL4* contains the *GAL4* coding sequence under the control of the *hsp70* promoter. Together with the data suggesting that *lilli* does not appear have a role in normal R8 or IOB development, it is likely that *lilli* does not play a specific role in the *sens* pathway, but rather affects transcription from the *lz-GAL4 hsp70* promoter. Since no other complementation group failed the secondary screens, they are likely to have a functionally significant interaction with *sens* and do not modify *ls* due to nonspecific interactions.

Mutations in *moleskin* suppress the bristle and ommatidial phenotypes generated by *ls* and result in reduced expression of ectopic *Sens*: Fifteen suppressor mutations form the largest complementation group identified in the screen. This group, *S(ls)2*, was mapped to the modifying deficiency *Df(3L)pbl-X1*. *Df(3L)pbl-X1* is a strong dominant suppressor of *ls* (see Figure 7, A and D). This deficiency uncovers cytological position 65F3–66B10. To identify a single gene within this deletion that conferred suppression, lethal alleles within this region were tested for modification of *ls*. Two previously identified embryonic lethal alleles of the gene *moleskin/dim-7*, *msk⁴* and *msk⁵* (BAKER *et al.* 2002), located at 66B6, suppress the *ls* phenotype and fail to complement *Df(3L)pbl-X1* (see Figure 7, B and E, and data not shown). All 15 EMS alleles also failed to complement the alleles *msk⁵* and *msk⁴* identifying this complementation group as *moleskin/DIM-7*. The *msk⁵* mutation is a slightly stronger suppressor of *ls* than two *S(ls)2* alleles we examined by SEM (Figure 7, B, C, E, and F, and data not shown). This is probably due to the fact that the *msk⁵* allele is a null due to a G > T point mutation that changes the second amino acid to a stop codon (BAKER *et al.* 2002). Since *msk⁵* is a molecularly characterized allele, and the molecular nature of the alleles isolated

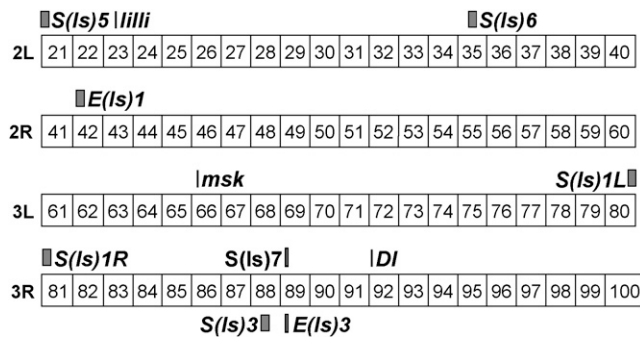


FIGURE 6.—Ten complementation groups are distributed across both arms of chromosomes 2 and 3. Boxes indicate the interval containing a complementation group. Complementation groups identified as known genes or groups mapped to narrow intervals are indicated with vertical bars. Note that *S(ls)1* could not be localized to one arm of chromosome 3 due to its extreme proximity to the centromere. Potential locations on either arm are denoted with an L or an R.

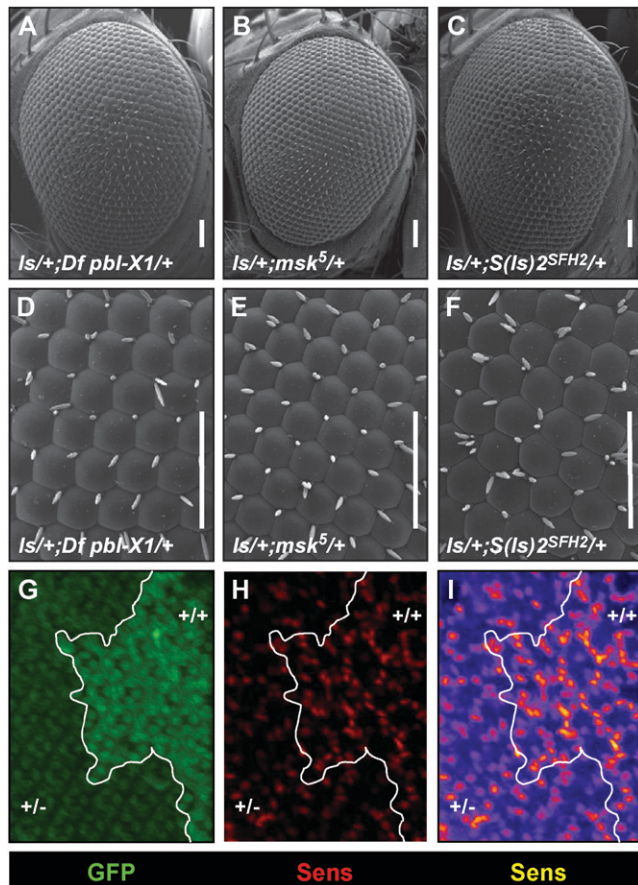


FIGURE 7.—*moleskin/dim-7* dominantly suppresses ectopic bristle formation and reduces ectopic Sens expression. *Df pbl-X1* uncovers *moleskin/dim-7* and was identified as a dominant suppressor of the *ls* phenotype (compare A and D with Figure 1, D and E). A previously identified mutation in *msk* (*msk⁵*) fails to complement the deficiency (data not shown) and also suppresses the *ls* phenotype (B and E). Fifteen EMS mutants were identified as alleles of *msk*; these also suppress the *ls* phenotype (C and F and data not shown). Consistent with this observation, ectopic expression of Sens in 48-hr APF discs heterozygous for *msk⁵* (+/–) is reduced compared to that in the wild-type twispot (+/+) (G–I). G shows GFP staining in green. The twispot boundary is outlined in white. Sens staining is shown in red (H). To illustrate the differences in Sens staining between the twispot and heterozygous tissue, pixel intensity from H was mapped to a heat scale (I); dim pixels are dark blue and bright pixels are yellow. Genotype of (+/+) is *ls/+; ubi-GFP, FRT80B/ubi-GFP, FRT80B*. Genotype of (+/–) is *ls/+; ubi-GFP, FRT80B/msk⁵, FRT80B*.

from the screen are not known, we decided to pursue further analysis of the interaction between *sens* and *msk* with this allele. Adult EGUF SEM and sectioning phenotypes generated with *msk⁵* were also generated with two EMS-isolated alleles and the results were consistent (data not shown). Importantly, no allele of *msk* suppressed the phenotypes generated by the *C96-GAL4, UAS-hrs* or *lz-GAL4, UAS-ro* secondary screens (data not shown). This suggests that the suppression of *ls* by *msk* is due to a specific interaction of *msk* with the ectopic sens pathway.

Msk is a nuclear transport protein, or karyopherin, and is homologous to Importin 7. Importins are a family of proteins that act to transport macromolecules to and from the nucleus (GORLICH *et al.* 1997; STROM and WEIS 2001). In *Drosophila*, *moleskin* has been implicated in the nuclear transport of proteins such as pMAPK (LORENZEN *et al.* 2001; VRILAS *et al.* 2006) and Caudal (HAN *et al.* 2004). Sens is a 61-kDa protein (NOLO *et al.* 2001); this is just above the predicted size exclusion limit for passive entry into the nucleus (BEDNENKO *et al.* 2003; WEIS 2003). This raised the possibility that *msk* dominantly suppresses the *ls* phenotype by limiting the entry of Sens into the nucleus. This model leads to two predictions. First, in the *ls* background we should be able to detect less nuclear Sens in tissue heterozygous for *msk* than in tissue with two wild-type copies of *msk*. Second, Sens and Msk should physically interact.

To test the first prediction, we generated mosaic eyes that contain neighboring wild-type (*msk* +/+) or heterozygous (*msk* +/-) tissue in an otherwise *ls* background (Figure 7, G–I). We predicted that these two types of tissue would behave differently with respect to nuclear localization of Sens and that in the heterozygous (+/-) tissue there would be a decrease in the number of Sens-expressing cells and a decrease in the levels of nuclear Sens detected. When we looked at 48-hr APF eye discs, this is indeed what we found. In the *ls* background, there were fewer Sens-positive cells in *msk* heterozygous (+/-) tissue than in wild-type tissue (+/+) ($P < 0.014$; see MATERIALS AND METHODS). This represented an average 1.5-fold difference between the number of Sens-positive cells per square micrometer in wild-type *vs.* heterozygous tissue (standard deviation = 0.22). In addition, in all images examined Sens expression in heterozygous cells was less intense than in wild-type cells (Figure 7, G–I). An intensity map of Sens staining in the *ls* background in either twispot (+/+) or heterozygous (+/-) *msk* tissue is shown in Figure 7I. Low levels of Sens staining are shown in blue, mid-range levels in red, and high levels in yellow. Figure 7I illustrates that more intense Sens staining in individual cells occurs in the unmodified twispots than in the heterozygous tissue. The simplest interpretation of these data is that in heterozygous *msk* tissue, nuclear entry of Sens or some factor necessary for ectopic Sens expression is decreased. With decreased nuclear transport, Sens function is reduced, and its ability to recruit cells to the bristle fate is decreased. This is consistent with the screen results that found strong functional suppression of the *ls* phenotype in heterozygous *msk* tissue. In contrast, control experiments with tissue lacking ectopic Sens (*i.e.*, non-*ls* background) did not reveal a difference in Sens nuclear expression in (+/+) *vs.* (+/-) tissue (data not shown). In addition, bristles are formed normally in *msk* heterozygous tissue (data not shown). These data suggest that the amount of Msk function required to maintain wild-type levels of Sens function is

lower than the amount of import required to produce the *ls* phenotype.

To test the second prediction, a direct interaction between Msk and Sens, GST-tagged Msk was generated and tested for its ability to pull down *in vitro* translated and transcribed Sens. Despite testing a range of wash conditions, a specific interaction between Msk and Sens could not be identified (data not shown). It is possible that the interaction is not direct or may require a cofactor that was not provided in the binding buffer. Thus, although a reduction in Sens nuclear import may contribute to the effect on staining and the suppression of the *ls* phenotype identified in the screen, it is more likely that Msk imports some other factor required for normal bristle development or Senseless pathway function.

moleskin is required for normal eye development:

Interactions detected in modifier screens do not always

accurately represent the biological processes present during normal development. Therefore, we next set out to assess the effect of loss of Msk on normal neural development and Sens function in the eye. To study the effect of a complete loss of Msk on normal development, we attempted to generate homozygous mutant eye tissue using mitotic recombination. Unfortunately, we found that *msk* clones are rare and small in larval eye tissue and are not detectable in pupal or adult tissue even when clones are made with a Minute chromosome (data not shown). This is consistent with previous observations in the wing and eye (BAKER *et al.* 2002; VRAILAS *et al.* 2006). The small clone size could be due to the inability of *msk* null tissue to compete with heterozygous tissue. To circumvent this problem, we made whole *msk* mutant eyes using the *ey-GAL4*, *UAS-flp* (EGUF) system, which removes competing heterozygous and wild-type cells due to the presence of the *GMR-hid* transgene (STOWERS and SCHWARZ 1999). Using this system, small, disorganized adult eyes that are mutant for *msk*⁵ are recovered (compare Figure 8, A and B to D and E). Examination of the adult eye morphology by SEM reveals ommatidial fusions, irregular ommatidial shape and size, and occasional bristle loss or duplication (compare Figure 8B to 8E). IOB loss and duplication as well as ommatidial fusions are also apparent in 48-hr APF discs stained with anti-Armadillo antibody (compare Figure 8G to 8H). In addition, Armadillo staining reveals that all cell types are affected by the loss of *msk*. Some ommatidia have clusters containing more than four cone cells (white arrow in Figure 8H), but others with only two or three cone cells are observed, and many

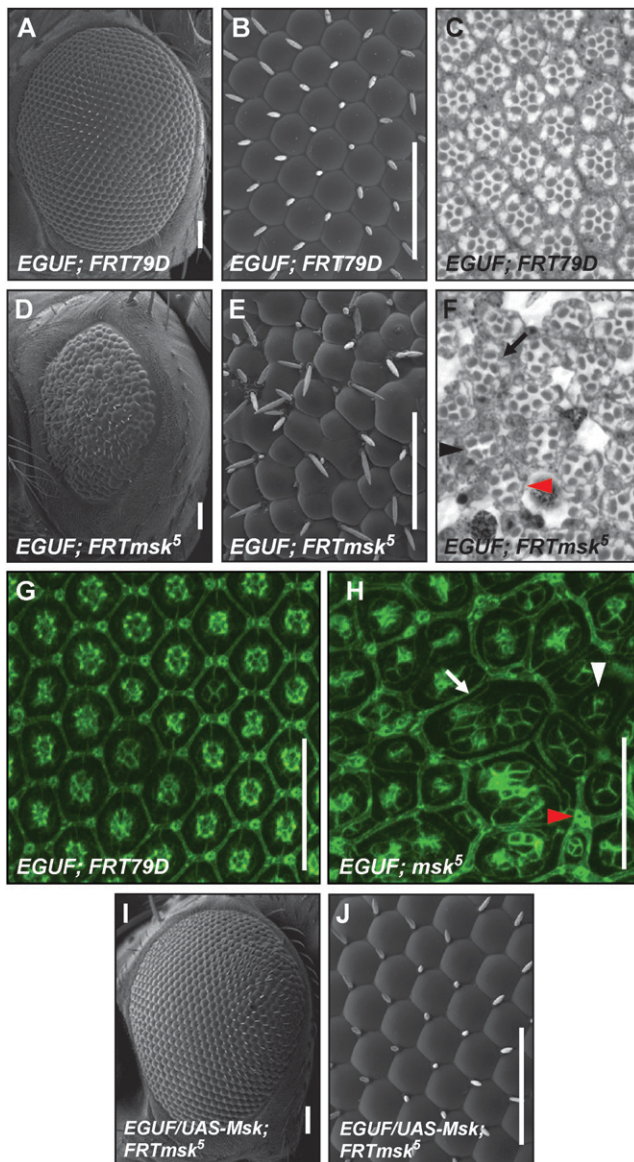


FIGURE 8.—*moleskin/dim-7* is required for normal eye development. An eye made entirely mutant for *msk*⁵ using the EGUF system is small and rough when compared with the control (D vs. A). At higher magnification ommatidial fusions and disruptions of the normal arrangement of bristles can be seen (compare E with B). Thin plastic sections of the EGUF; *msk*⁵ eye reveal a general disruption of normal ommatidial organization and numerous abnormal rhabdomeres (compare F with C). Some ommatidia have a normal number and arrangement of rhabdomeres (black arrow in F) while others have a decreased number of rhabdomeres per ommatidium (black arrowhead in F). Some ommatidia lack any small rhabdomeres (red arrowhead in F). This loss was verified by serial section (data not shown). At 48 hr APF the control eye has already formed the crystalline lattice seen in the adult eye (G). In the EGUF; *msk*⁵ eye, the cellular organization is clearly disrupted at 48 hr APF (H). The number of cone cells per ommatidium is often decreased to three (white arrowhead in H). Ommatidial fusions are seen where both primary and secondary pigment cells are absent (white arrow in H). Evidence of occasional bristle duplications seen by SEM in E are also apparent in the pupal disc (red arrowhead in H). The EGUF; *msk*⁵ mutant phenotype is rescued with the addition of UAS-*msk* (I and J). Bars, 50 μm. Genotype of EGUF; *msk*⁵ is *w*; *ey-GAL4*, *UAS-flp*/+; *msk*⁵, *FRT79D*/*GMR-hid*, *Cl FRT79D*. Genotype of EGUF control is *w*; *ey-GAL4*, *UAS-flp*/+; *FRT79D*/*GMR-hid*, *Cl FRT79D*. Genotype of EGUF/*UAS-msk*; *FRTmsk*⁵ is *w*; *ey-GAL4*, *UAS-flp*/*UAS-msk*; *msk*⁵, *FRT79D*/*GMR-hid*, *Cl FRT79D*.

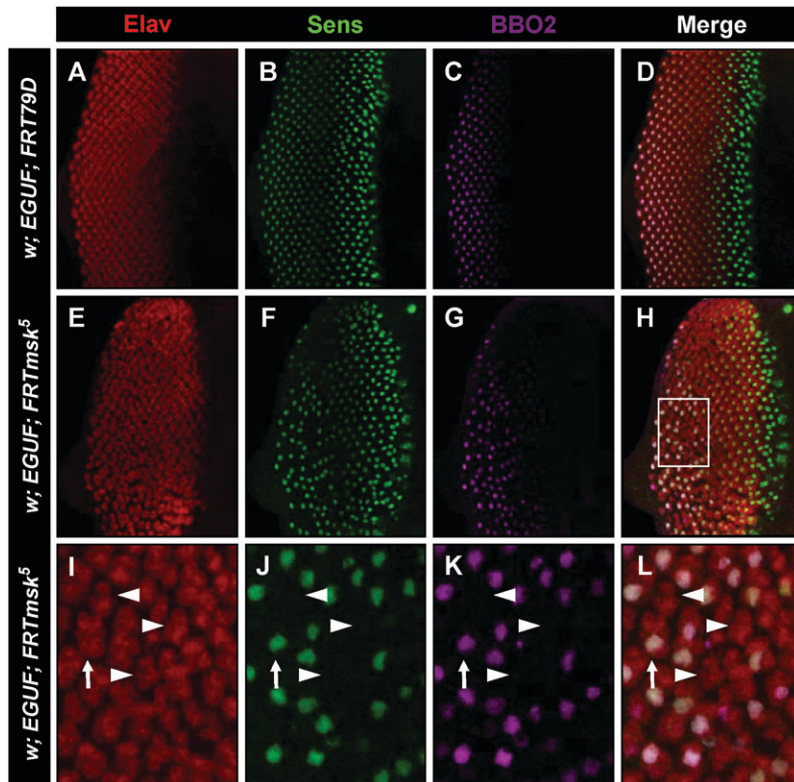


FIGURE 9.—Loss of *moleskin/dim-7* function leads to the sporadic loss of R8 photoreceptors. Expression of Elav (A, E, and I), Sens (B, F, and J), and *BBO2* (C, G, and K) in control (A–D) *vs.* *EGUF; msk⁵* (E–L) third instar larval eye discs is shown. I–L show a magnified view of the boxed area in H. Elav staining in wild-type third instar larval discs reveals the emerging crystalline order seen in the adult eye (A). Each wild-type Elav-positive cluster contains a Sens-positive R8 (B). The *P{lacW}* enhancer trap *BBO2* is a later marker of the R8 photoreceptor. More mature R8 photoreceptors express both Sens and *BBO2* (D and arrow in I–K). In contrast, the *EGUF; msk⁵* eye (E–H) shows disruption of the wild-type organization with uneven spacing between Elav-positive clusters. Furthermore, some Elav-positive clusters lack Sens and *BBO2* (arrowheads in I–L). Genotype of *w; EGUF; msk⁵* is *w; ey-GAL4,UAS-flp/BBO2; msk5, FRT79D/GMR-hid, ClFRT79D*. Genotype of *w; EGUF; FRT79D* is *w; ey-GAL4,UAS-flp/BBO2; FRT79D/GMR-hid, ClFRT79D*.

of these cone cells are an abnormal size. Primary pigment cells are often enlarged and abnormally shaped. Secondary and tertiary pigment cell number is often drastically reduced, as shown by multiple ommatidial fusions (arrow in Figure 8H). We examined Armadillo staining at earlier stages (third larval instar, 6 and 24 hr APF; data not shown), to determine at what stage loss of *msk* led to abnormal development. By third instar, abnormalities can already be seen in the spacing of ommatidial clusters as they emerge from the furrow (data not shown). To verify that the *EGUF; msk⁵* phenotype is due specifically to the loss of *msk*, *UAS-msk* was expressed in *EGUF; msk⁵* eyes (compare Figure 8, D and E to I and J). Eye development was rescued with the exception of minor disorganization at the posterior margin.

Loss of *moleskin* leads to the sporadic loss of R8 photoreceptors: Thin plastic sections of the *EGUF; msk⁵* eyes also demonstrate severe disorganization (Figure 8, C and F). There are occasional ommatidia that form with the normal number of rhabdomeres in the stereotypical trapezoidal pattern (arrow in Figure 8F), but most have fewer than normal numbers of rhabdomeres (arrowhead in Figure 8F). Normal rhabdomere morphology is also disrupted: some are abnormal in shape, appearing rectangular or oblong rather than the usual, more round aspect seen in wild-type eyes. In addition, multiple ommatidia lacking small rhabdomeres are seen. The small rhabdomeres of R7 and R8 are normally located in the center of each ommatidium, but at different depths. To test for the presence of both small rhabdomeres, serial sectioning of *EGUF; msk⁵* eyes was

performed through the entire thickness of the retina. We found that 22% of ommatidia in the *EGUF; msk⁵* eye were completely missing small rhabdomeres (representative shown by red arrowhead in Figure 8F). This is interesting because this effect on small rhabdomeres is reminiscent of the *sens* mutant phenotype, which shows a complete loss of small rhabdomeres.

To further explore this phenotypic link between *msk* and *sens*, we investigated the effect of loss of *msk* on the expression of Sens in the developing R8 photoreceptor. Sens is required in the R8 cell to maintain the R8 fate by repressing the expression of the homeodomain transcription factor Rough (FRANKFORT *et al.* 2001). In the absence of Sens, the R8 adopts the R2,5 cell fate. Consistent with the sporadic loss of adult R8's, we found that Sens staining was lost sporadically throughout the *EGUF; msk⁵* larval disc (Figure 9, F and J; compare to Figure 9B). This incomplete loss of Sens expression in developing clusters of the homozygous *msk* disc was surprising considering the strong effect of heterozygous *msk* loss on the screen phenotype. This indicates that *msk* is not the sole transport factor utilized by the Sens pathway and suggests that *msk* may affect the *sens* pathway differently in R8 and IOB development. A previous study exploring the role of *msk* in *Drosophila* eye development did not detect an effect of *msk* loss on Sens expression in R8 development (VRAILAS *et al.* 2006). This is not surprising due to the sporadic effect of *Msk* loss on Sens expression. Consistent with our attempts to make mosaic *msk* eyes, Vrailas *et al.* were able to generate small clones of only a few cell widths. Detecting Sens loss with

this method would require observation of potentially hundreds of such small clones. We are able to detect this interaction due to the large number of mutant ommatidia generated with the *EGUF* system. To determine if the loss of Sens staining in *msk* mutants also leads to the loss of the R8 fate, we tested for the expression of *BBO2*, an enhancer trap known to be expressed later in R8 development. *BBO2* is normally expressed in the R8 photoreceptor by column 11–12 (Figure 9C) (HART *et al.* 1990). In *sens* null tissue, *BBO2* expression is lost in the putative R8 (FRANKFORT *et al.* 2001). We found that this marker is also lost in *EGUF; msk⁵* cells that also lack Sens expression (Figure 9, G and K). Therefore, loss of *msk* leads to sporadic loss of R8 photoreceptors during larval development.

There are three potential mechanisms for loss of the R8 photoreceptor: the R8 is never specified, the R8 is specified but then dies, or the R8 is specified but then changes fate. Our lab previously explored each of these possibilities while defining the *sens* mutant phenotype and determined that the presumptive R8 changed fate to an outer R2,5-type photoreceptor. We tested these possibilities in *msk* mutant discs to determine the mechanism for R8 loss in this genotype. In the case where an R8 is never specified, such as in an *atonal* mutant, no photoreceptors develop due to the lack of the EGFR ligand *spitz*, which is normally produced by R8 (TIO *et al.* 1994; TIO and MOSES 1997). Therefore, if R8 is never specified, no other photoreceptors will develop or be detectable by staining for the neuronal antigen Elav (embryonic lethal, abnormal vision). As seen in Figure 9L, there are multiple Elav-positive, Sens-negative clusters in *msk* tissue, suggesting that the R8 cell was specified in the *msk* mutant and was able to initiate outer photoreceptor development before it either died or changed fate.

Due to the similarity of the *msk* and *sens* mutant phenotypes and the genetic interaction detected between *msk* and *sens*, we hypothesized that the *msk* R8 phenotype may be due to an effect on *sens* expression during normal development. This hypothesis predicts that the missing R8's in *EGUF; msk⁵* eyes also switch fate to an R2,5-like photoreceptor. This hallmark of the *sens* phenotype is identified in the third instar disc by an Elav-positive cluster that lacks a Sens- or a *BBO2*-positive cell, but gains a third *RM104*-positive cell (FRANKFORT *et al.* 2001). *RM104* is an enhancer trap specifically expressed in R2/5 in wild-type tissue (arrowheads in Figure 10, C and D). As seen in the *sens* mutant, *EGUF; msk⁵* ommatidia lacking Sens expression also have three cells expressing *RM104* (arrowheads in Figure 10, G and H). Therefore, loss of *msk* can lead to an early cell fate switch of R8 to an R2,5 photoreceptor and sporadically phenocopies the *sens* loss-of-function photoreceptor phenotype.

These data support a model where, in some developing clusters in *msk* mutant tissue, the level of nuclear

transport stochastically fails to reach a critical threshold and leads to loss of both Sens expression and the R8 cell fate. However, the causal relationship and order of events is not clear: Does the loss of Sens lead to the loss of R8 fate, or is there an upstream effect on R8 fate that is the cause of the loss of Sens expression? To determine which event occurs first, we examined R8-less clusters for expression of the early R8 marker *sca-lacZ*. Previously, we demonstrated that in *sens* clones, *sca-lacZ* remains on and marks the cell that should have developed as an R8 (FRANKFORT *et al.* 2001). To determine when *msk* is required for R8 specification, we tested the Sens-negative clusters in *EGUF; msk⁵* eyes for the expression of *sca-lacZ* (Figure 10, M–P). We found that clusters lacking Sens also lack *sca-lacZ* expression, suggesting that the R8 fate is compromised before the onset of Sens expression. The presence of three *RM104*-positive cells in R8-less Elav-positive clusters suggests that loss of *msk* causes a sporadic failure of R8 development that occurs after the initial specification by Atonal, but before the onset of *sca-lacZ* and Sens expression.

Superficially, loss of *msk* resembles the *sens* mutant phenotype with incomplete penetrance. However, these data also suggest that Msk has an independent role upstream of *sens* in normal eye development where it may be required to import other factors important in R8 fate maintenance. Interestingly, this is the first phenotype to identify a genetically separable developmental stage between Senseless and Atonal.

DISCUSSION

Sens, along with its homologs Gfi-1 and Pag-3, comprises a conserved family of proteins required for normal neural development. In *Drosophila*, *sens* is both necessary and sufficient for development of the PNS. In mice, loss of *Gfi-1* leads to neurodegeneration of cerebellar Purkinje cells and sensorineural deafness due to loss of inner ear hair cells (WALLIS *et al.* 2003; TSUDA *et al.* 2005). Despite the obvious importance of the GPS proteins in normal neural development and their place near the top of the neuronal development cascade, few targets of these proteins in the process of neurogenesis are known. To identify members of this pathway required in neurogenesis, we performed an F₁ dominant modifier screen using an ectopic Sens phenotype in *Drosophila*. We took advantage of a dominant, modifiable phenotype generated by ectopic expression of Sens in undifferentiated cells posterior to the morphogenetic furrow. This ectopic Sens led to the recruitment of undifferentiated cells to the bristle fate.

We report here on both known and novel genes that have been identified as potential members of the *sens* pathway by their ability to modify an ectopic Sens phenotype. The Notch signaling pathway is known to regulate Sens function during the resolution of the proneural cluster (JAFAR-NEJAD *et al.* 2003). This interaction was

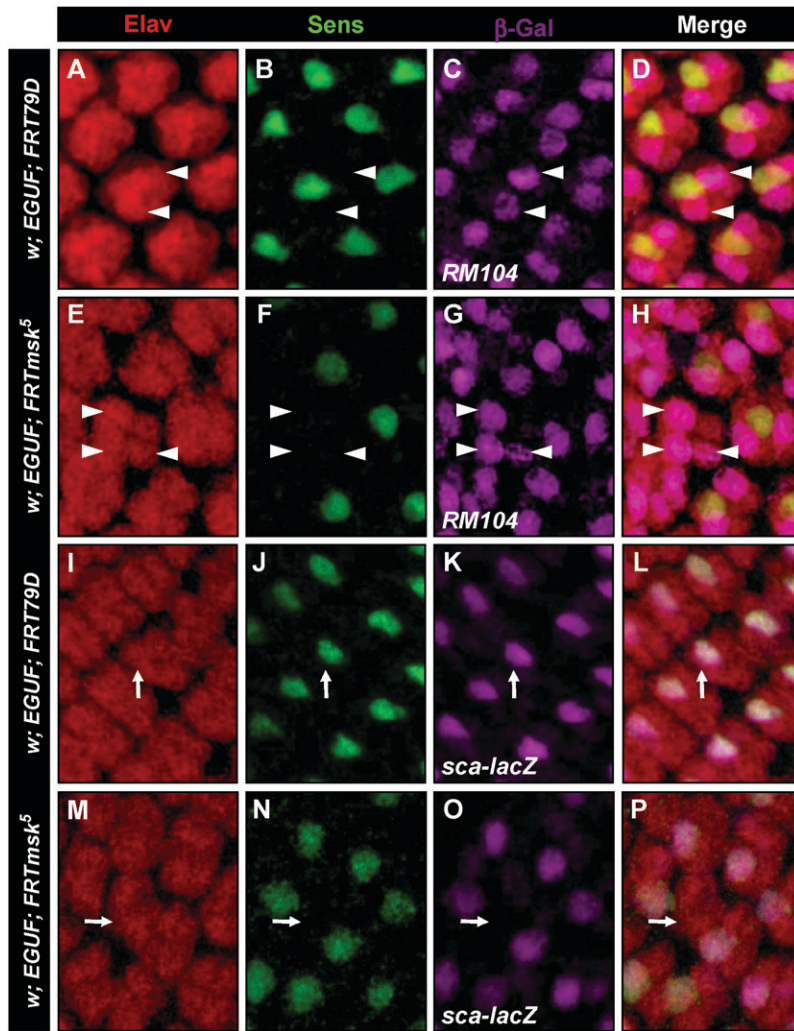


FIGURE 10.—The mechanism of R8 cell loss is through a cell fate change to R2,5 that occurs earlier than in *sens* mutants. In a control eye (A–D), Sens (green) is expressed in one cell per cluster and the enhancer trap *RM104* (purple) is expressed in the R2 and R5 cells (arrowheads in A–D). In the *EGUF; msk⁵* disc (E–H), occasional clusters lacking Sens express *RM104* in more than two cells (arrowheads in G and H). Elav staining is shown in red to illustrate ommatidial clusters. The enhancer trap *sca-lacZ* is normally expressed in R8 cells and coincides with Sens expression (I–L). In *EGUF; msk⁵* eyes, clusters lacking Sens expression also lack *sca-lacZ* expression. See MATERIALS AND METHODS for genotypes.

identified in our screen by the ability of heterozygous loss of *Dl* to enhance the ectopic Sens phenotype. The nuclear import gene *moleskin* (*msk*) was able to strongly suppress the effect of ectopic Sens. We also show that *msk* plays a role in normal eye development and R8 photoreceptor differentiation. Identification of the genes that are represented in the remaining complementation groups will lead to a better understanding of the GPS pathway and normal neural development. It is likely that the remaining complementation groups represent components of the Sens pathway due to their specific effect on *ls* and not the secondary screens as well as their requirement for normal bristle development in adult thoracic clones (to be reported elsewhere). Further characterization of these genes will offer new insight into the highly conserved Sens pathway.

Alleles of *msk* were found to be suppressors of *ls* with the highest frequency of any complementation group in the EMS screen. Usually such high representation of alleles indicates that the gene has an important role in the phenotype being tested and/or is readily mutagenized. The results presented here suggest a model in which Msk plays a role in the *sens* pathway. Our initial

observations of the effect of Msk on the *ls* phenotype suggested that Msk was needed to maintain high levels of Sens expression. It is possible that in this ectopic situation, Msk contributes to Sens import, but more likely Msk contributes to Sens expression indirectly by importing another component of the pathway that regulates Sens expression. Characterization of the *EGUF; msk* phenotype strongly suggests that Msk is not the only import factor involved in the Sens pathway during normal development. Clearly, there is functional redundancy with another importin since complete loss of Msk function during early eye development does not remove Sens expression in all R8 cells. In third instar discs, Msk appears to play a role in the maintenance of the R8 cell fate very early in development. Little is known about the early stages of R8 differentiation after specification by Atonal. Previous work on R8 specification and development outlined a hierarchy of events in which Atonal is expressed first and appears to simultaneously activate expression of the downstream targets *sens* and *sca-lacZ* (MŁODZIK *et al.* 1990; FRANKFORT *et al.* 2001). Work on the *sens* phenotype determined that *sca-lacZ* expression is still present in *sens* clones, thereby establishing an

epistatic relationship between *sca-lacZ* expression and *sens* (FRANKFORT *et al.* 2001). Our data indicate that there is yet another step in the relationship between Atonal and these two downstream factors. Our data suggest that in the *msk* eye, after specification of the R8 by Atonal but before the onset of *sca-lacZ* expression, R8 development is disrupted in some clusters, leading to an R2,5 fate switch. This is the first genetic evidence for factors positioned between *ato* and *sca-LacZ/sens*.

Nuclear transport is required for the viability of all cells. Interestingly, the loss or decrease in function of some importins can cause specific defects during development. For example, the nuclear exportin Dcas is required for the export of Importin $\alpha 3$ in *Drosophila* (TEKOTTE and DAVIS 2002). While null mutants in *dcas* are not viable, hypomorphs lead to specific cell fate changes in mechanosensory bristles. This phenotype is likely due to extreme sensitivity of Notch signaling to disruption of nuclear transport of one of its pathway members by Importin $\alpha 3$. It is possible that the Msk/Sens interaction was detectable for a similar reason. In the Sens gain-of-function situation, the high level of Sens required to generate ectopic bristles is very sensitive to decreased Msk levels, while during wild-type SOP differentiation, Sens is far less sensitive to Msk levels and exhibits only sporadic effects.

One question still remains: How does the *EGUF; msk* eye survive at all given the important cargo that Msk is known to transport? The functional redundancy in the Importin family likely provides the cell with enough transport for survival and development in the absence of Msk. However, this idea raises a new question: Why did we identify only Msk in our screen and no other importins? We propose a model in which Msk is the key importin utilized by the cell for high levels of signaling. The *ls* phenotype requires high levels of signaling to generate ectopic bristles, and this model would explain why we detected an effect with Msk and no other importin. The model does not preclude the ability of other importins to provide transport redundancy for Msk cargos, and in fact we see evidence for this redundancy in the ability of the *EGUF; msk* eye to survive and produce some normal ommatidia. Another importin must have the ability to import some level of Sens, pMAPK, and other unidentified factors into the nucleus. The recent article by VRAILAS *et al.* (2006) provides data that indirectly support such a model for the role of Msk. In VRAILAS *et al.* (2006) the authors show that in the Atonal intermediate groups within the morphogenetic furrow, Msk must be sequestered away from the nucleus to prevent the very high levels of cytoplasmic pMAPK from entering the nucleus. Although they do not test if other nuclear importins are also sequestered to block pMAPK nuclear entry, they do show that overexpression of Msk in the intermediate groups allows pMAPK to enter the nucleus and affect nuclear signaling. The fact that the cell needs to sequester Msk to prevent high levels of

EGFR pathway signaling supports a model in which Msk is important for high levels of signaling.

It has been suggested in other developmental systems that importins are part of a mechanism that regulates the nuclear protein composition of transcription factors and chromatin remodeling factors (HOGARTH *et al.* 2005). In *Drosophila*, Msk has been shown to import two other developmentally significant cargos, pMAPK and Caudal (LORENZEN *et al.* 2001; HAN *et al.* 2004; VRAILAS *et al.* 2006). In addition to these previously defined roles, here we show additional data that Msk and nucleocytoplasmic transport play an important role in Sens expression and R8 development. Perhaps more importantly, the fact that abnormalities seen in *msk* mutant eye discs arise between Atonal and Senseless expression suggests roles for as-yet undiscovered factors and new modes of regulation in this critical pathway.

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