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Drosophila JAB1/CSN5 Acts in Photoreceptor Cells to Induce Glial Cells

Greg S.B. Suh,[1] Burkhard Poeck,[1,2] Tanguy Chouard,[1] Efrat Oron,[3] Daniel Segal,[4] Daniel A. Chamovitz,[3] and S. Lawrence Zipursky[1,5]

[1] Department of Biological Chemistry
Howard Hughes Medical Institute
The School of Medicine
University of California, Los Angeles
Los Angeles, California 90095

[2] Lehrstuhl für Entwicklungsbiologie
Institut für Zoologie
Universität Regensburg
Universitätsstr. 31
93053 Regensburg
Germany

[3] Department of Plant Sciences

[4] Department of Molecular Microbiology and Biotechnology
Tel Aviv University
69978 Tel Aviv
Israel

Summary

Different classes of photoreceptor neurons (R cells) in the *Drosophila* compound eye form connections in different optic ganglia. The R1-R6 subclass connects to the first optic ganglion, the lamina, and relies upon glial cells as intermediate targets. Conversely, R cells promote glial cell development including migration of glial cells into the target region. Here, we show that the JAB1/CSN5 subunit of the COP9 signalosome complex is expressed in R cells, accumulates in the developing optic lobe neuropil, and through the analysis of a unique set of missense mutations, is required in R cells to induce lamina glial cell migration. In these CSN5 alleles, R1-R6 targeting is disrupted. Genetic analysis of protein null alleles further revealed that the COP9 signalosome is required at an earlier stage of development for R cell differentiation.

Introduction

The construction of a precise pattern of neuronal connections requires multiple cellular interactions. Growth cones are guided to their targets by a specific sequence of spatially regulated cues and engage in a complex dialog with their targets. Growth cones respond to specific signals in the target region and, in some cases, produce signals that regulate target development. Such anterograde signals play a key role in regulating the formation of precise patterns of connections between the photoreceptor neurons (R cells) in the compound eye of the fly and their targets in the brain (Salecker et al., 1998).

The compound eye of the fly contains some 750–800 simple eyes called ommatidia. Each ommatidium contains eight R cells. The R1-R6 axons project to a unique set of targets in the first optic ganglion, the lamina, while R7 and R8 terminate in two distinct layers in the second optic ganglion, the medulla. Genetic analysis has led to the identification of genes encoding growth cone receptors (Garrity et al., 1999; Newsome et al., 2000b; Lee et al., 2001), signaling molecules (Garrity et al., 1996; Hing et al., 1999; Newsome et al., 2000a), and nuclear factors (Rao et al., 2000; Senti et al., 2000) important for the elaboration of these connections.

A striking feature of the fly visual system is its modularity. The number of ommatidia precisely matches the number of postsynaptic units in the lamina (cartridges) and in the medulla (columns). Studies by Power (1943) and Meyerowitz and Kankel (1978) argued that anterograde signals from R cell afferents play a key role in determining target size. Subsequent studies revealed that R cell afferents control the number of lamina neurons and glial cells (Salecker et al., 1998).

R cells produce two distinct signals that regulate lamina neuronal differentiation. The first signal, Hedgehog, is transported down R cell axons and induces lamina neuronal precursor cells to undergo their final cell division (Huang and Kunes, 1996). A second signal, Spitz (an EGF receptor ligand), is required to induce differentiation of lamina neurons (Huang et al., 1998). R cell expression of Hedgehog and Spitz also plays a crucial role in regulating patterning in the eye (e.g., Ma et al., 1993; Heberlein et al., 1993; Tio and Moses, 1997). It has been proposed that a third signal, yet to be identified, induces the differentiation of lamina glial cells. These cells are intermediate targets for R1-R6 neurons; in their absence, R1-R6 cells project into the medulla (Poeck et al., 2001).

In this paper, we present evidence that a component of the COP9 signalosome (CSN) (Chamovitz et al., 1996) is required in R cell afferents for lamina glial development and R1-R6 targeting. The CSN is an evolutionarily conserved protein complex that contains eight core subunits termed CSN1 through CSN8, in order of decreasing subunit size. While the exact biochemical functioning of the CSN is unclear, the complex or its individual subunits have been implicated in diverse signaling pathways in plants and animals (reviewed in Chamovitz and Segal, 2001; Kim et al., 2001). Recent evidence has implicated major roles for the CSN in regulating protein degradation, either through direct regulation of E3 ubiquitin-ligases (Lyapina et al., 2001; Zhou et al., 2001; Schwechheimer et al., 2001) or through regulation of the phosphorylation of specific substrates such as p53 and c-Jun (Naumann et al., 1999; Bech-Otschir et al., 2001). Three missense mutations in an N-terminal domain of the JAB1/CSN5 subunit of the COP9 signalosome selectively disrupt R1-R6 targeting and result in marked defects in glial cell development within the target region. These alleles do not disrupt R cell differentiation. JAB1/CSN5 is localized to the lamina neuropil comprising R cell growth cones that lie adjacent to lamina glial cells, supporting an intimate role for JAB1/CSN5 in mediating interactions be-

[5] Correspondence: zipursky@hhmi.ucla.edu

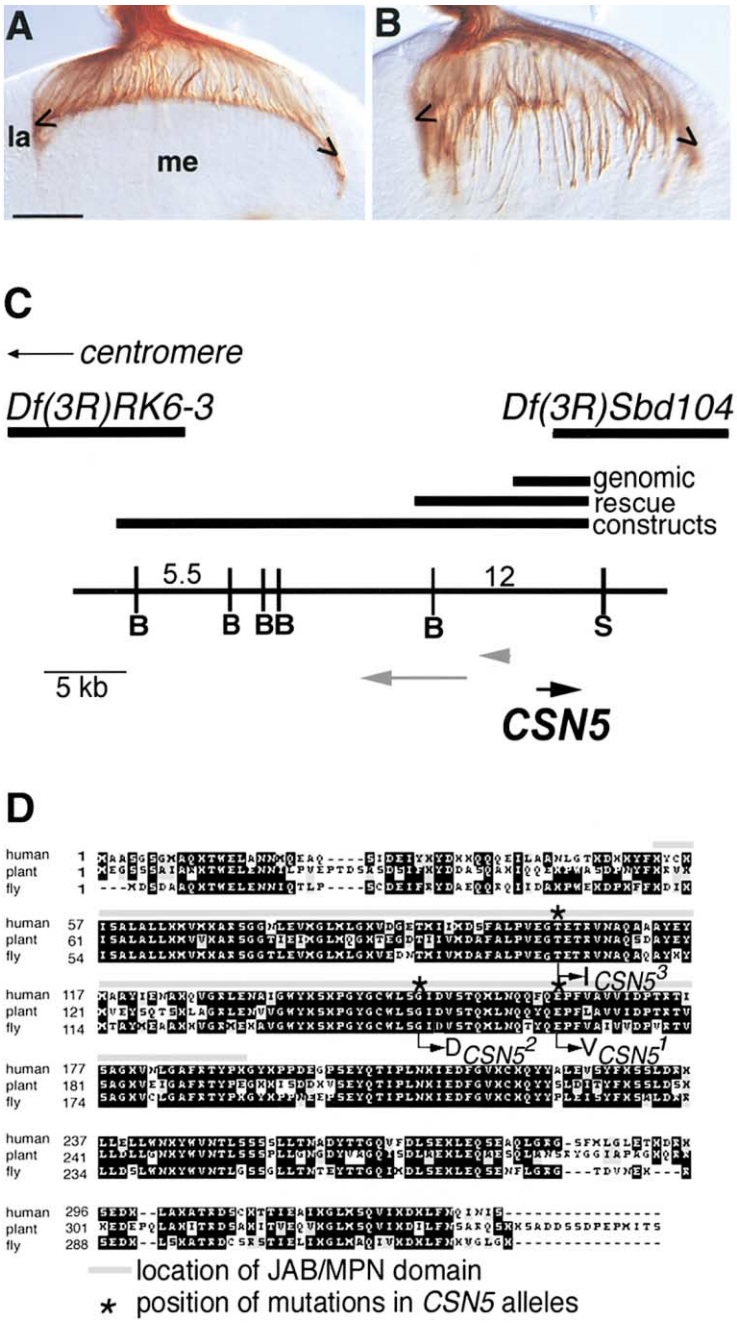

Figure 1. R Cell Axon Targeting Is Disrupted in *quo* Mutants

Note that through subsequent molecular analysis *quo* was renamed *CSN5*.

(A and B) The projections of a subset of R cell axons, R2-R5, in third instar larval eye-brain complexes were assessed using *Ro-τlacZ*. Scale = 50 μm. (A) In wild-type, R2-R5 axons terminate in the lamina. (B) In *quo*^{1}/*Df(3R)RK6-3*, many R2-R5 axons fail to terminate in the lamina (la) and instead, project into the medulla (me). The brackets indicate the lamina plexus, containing R1-R6 growth cones in wild-type.

(C) Positional cloning of the *quo* locus. The *quo* mutations map to a chromosomal region defined by the proximal breakpoint of *Df(3R)RK6-3* and the distal breakpoint of *Df(3R)Sbd104*. Genomic Southern blots showed that the 5.5 Kb BamHI and 12 kb BamHI/Sau3A fragments hybridize to the breakpoints in *Df(3R)RK6-3* and *Df(3R)Sbd104*, respectively. Three genomic rescue constructs containing the *CSN5* transcription unit and a heat shock *CSN5* cDNA transgene rescue both the *quo* connectivity and lethality phenotypes (see Experimental Procedures).

(D) Alignment of *Drosophila* JAB1/CSN5 with human and plant (*Arabidopsis thaliana*) JAB1/CSN5 reveals extensive homology. Asterisks indicate locations of missense mutations: *CSN5*^{1}(E_{160}→V), *CSN5*^{2}(G_{146}→D), and *CSN5*^{3}(T_{99}→I). The missense mutations are found in a region called the JAB/MPN domain (SMART Program; EMBL).

tween growth cones and lamina glial cells. JAB1/CSN5 also plays a critical role in an early function in R cell development, as was revealed through the isolation and characterization of null alleles deleting the JAB1/CSN5 coding sequence.

Results

R Cell Projections Are Disrupted in *quo* Mutants

A single loss-of-function mutation, initially designated *quo*^{1}, was identified in an ethyl methane sulfonate (EMS) screen for defects in R cell connections. Two additional EMS-induced alleles were identified, *quo*^{2} and *quo*^{3}, from a collection of lethal mutations generated in the 89C/D region (Sanchez-Herrero et al., 1985). *quo*^{1} mutant animals die as larvae or pupae, while *quo*^{2} and *quo*^{3} phenotypes were less severe with a lethal phase later during pupal development. In some cases, *quo*^{2}/*quo*^{3} heterozygotes survived to adulthood. These animals were morphologically normal, but were uncoordinated and sluggish, and died a few days after eclosion.

In *quo*^{1}, *quo*^{2}, and *quo*^{3} mutant larvae, many R1-R6 axons fail to terminate in the lamina but instead project through this region into the medulla (Figure 1B). This defect in target specificity was revealed using a marker for a subset of R1-R6 axons (i.e., R2-R5), *Ro-τlacZ* (Garrity et al., 1999). In contrast to wild-type, approximately 60% of the R2-R5 neurons in *quo*^{1} projected into the

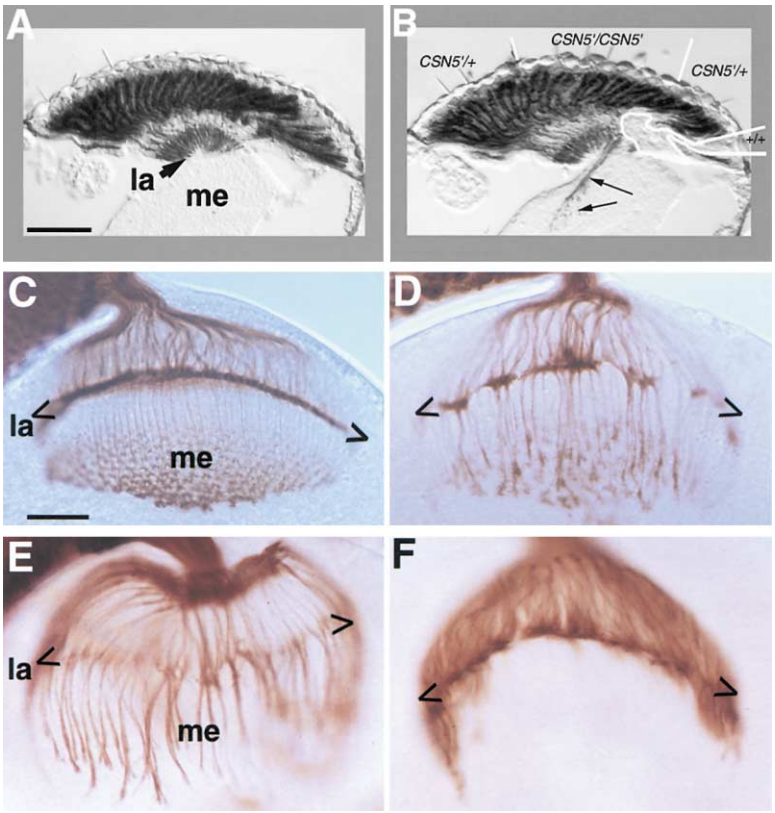

Figure 2. *CSN5* Is Required in R Cells for Targeting of R1-R6 Axons to the Lamina

(A and B) R1-R6 axons were visualized using the *Rh1-lacZ* marker in cryostat sections of adult heads. (A) In wild-type, lacZ-stained axons terminated in the lamina (arrow). (B) In mosaic animals, *CSN5^{1}* mutant R1-R6 axons projected into the medulla (see arrows). Mutant clones were generated using X-ray-induced mitotic recombination. Mutant tissue in the eye was scored prior to staining with anti-LacZ antibody. Since the *Rh1-lacZ* marker was recombined onto the same chromosomal arm as the *CSN5^{1}* allele, the twin spot (i.e., CSN5^{+}/CSN5^{+}, designated +/+) was not stained.

(C and D) R cell projection phenotypes in eye-brain complexes were visualized using mAb24B10. (C) Wild-type. (D) Abnormal projections were seen in mosaic animals in which mutant R cells project into a wild-type brain. The mosaic animals were generated as described in Experimental Procedures. This phenotype is similar to *CSN5^{1}/Df(3R)RK6-3* eye brain complexes stained with mAb24B10 (data not shown). Hence, the mistargeting phenotype in *CSN5* homozygotes is a consequence of loss of *CSN5* function in the eye.

(E and F) R2-R5 axons in third larval eye-brain complexes were visualized with using *Ro-τlacZ* as described in Figures 1A and 1B. (E) In *CSN5^{1}/Df(3R)RK6-3* mutants, R2-R5 axons do not properly terminate in the lamina. (F) Expression of *CSN5* cDNA in postmitotic neurons using *Elav*-gal4 to drive UAS-*CSN5* largely rescues the targeting phenotype.

Scale bar: (A and B) = 70 μm, (C–F) = 50 μm.

medulla. Mistargeting in homozygous *quo^{1}* mutants and in *quo^{1}* in *trans* to a deficiency was indistinguishable (data not shown). *quo^{2}* and *quo^{3}* phenotypes were qualitatively similar to, but less severe than, *quo^{1}*.

quo Encodes a *Drosophila* Homolog of JAB1/CSN5

Meiotic recombination and deficiency mapping localized *quo* to the cytological region between bands 89C7 and 89D1 (Figure 1C). Complementation tests placed *quo* between the proximal breakpoint of *Df(3R)RK6-3* and the distal breakpoint of *Df(3R)Sbd104*. A region of some 200 kb was isolated through positional cloning and approximately 20 kb of that region, defined by the two aforementioned breakpoints, demarcated the region containing the mutations. A panel of fragments was introduced into flies by P element-mediated transformation and tested for rescue of the *quo* connectivity defects and lethality. A genomic fragment of 6.6 kb rescued the mutant phenotype. A single open reading frame of 327 amino acids was identified within the fragment and cDNAs corresponding to it were isolated. A heat shock cDNA transgene rescued both lethality and the R cell targeting phenotype. NCBI Blast search results showed that this cDNA encoded a protein 65% and 74% identical to Jun-activation-domain binding protein1 (JAB1) (Claret et al., 1996) and subunit 5 of the *Arabidopsis* COP9 signalosome (CSN5) (Kwok et al., 1998), respectively (Figure 1D). Each of the three EMS alleles leads to specific amino acid substitutions in the JAB/MPN domain (as defined in the SMART program [EMBL]) in residues conserved between plant, fly, and human (Figure 1D). In the remainder of this manuscript, we refer to the *quo* gene (and its alleles) as *CSN5* and the Quo protein as JAB1/CSN5.

CSN5 Is Required in the Eye for R Cell Axon Targeting

To determine whether *CSN5* was required in R cell afferents or the brain, genetic mosaic analyses were undertaken. *CSN5^{1}* mutant patches in the retina, in otherwise heterozygous animals, were created by X-ray-induced mitotic recombination or by using FLP/FRT-mediated recombination driven by eye-specific expression of the FLP-recombinase (see Experimental Procedures). R1-R6 targeting in adult tissue was assessed using a marker specific for the projections of R1-R6 axons, *Rh1-lacZ*. Of 22 mosaic animals, 15 exhibited an R1-R6 mistargeting phenotype (Figure 2B). These results were confirmed using genetic mosaic analyses in developing eye-brain complexes by generating eye tissue homozygous for *CSN5^{1}* while the target was wild-type. These projections were assessed using a pan-R cell specific marker, mAb24B10. All animals of this genotype (n = 11) exhibited abnormal projections (Figure 2D). These results and the finding that the *CSN5* mutant phenotype was rescued by neuron-specific expression of a full-length cDNA (Figure 2F)

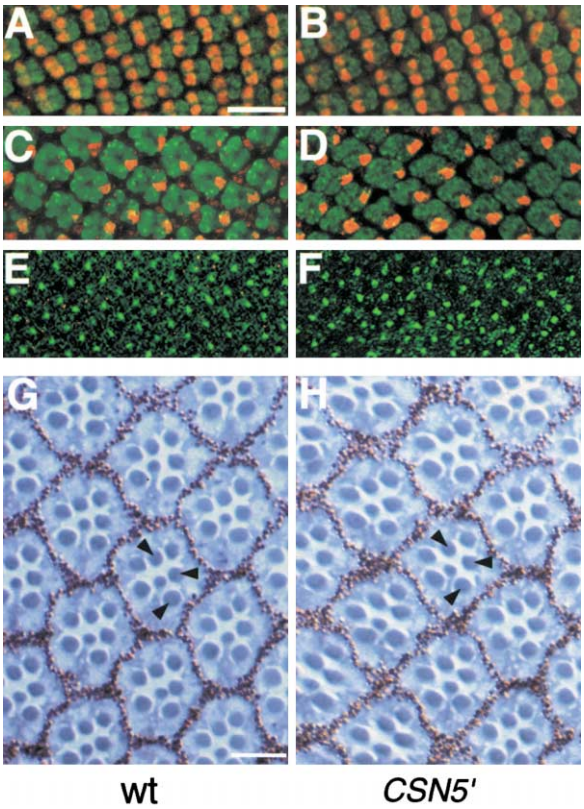

Figure 3. R Cell Fate Determination and Differentiation Are Normal in *CSN5[1]*

(A–F) Bar (A and B), Prospero (C and D), and Boss (E and F) were expressed in *CSN5[1]* eye discs as in wild-type. Bar (red) is expressed in R1 and R6 cells, Prospero (red) is expressed in R7 and cone cells (cone cells not shown in this preparation), and Boss is expressed in R8 cells.

(G and H) Plastic sections of adult wild-type and *CSN5[1]* mutant eyes (generated by ey-Flp-induced mitotic recombination) stained with toluidine blue. The number and position of R cell bodies, the morphology of the photosensitive structures (rhabdomeres, indicated by arrowheads), and the overall ommatidial array in *CSN5[1]* were indistinguishable from wild-type.

Scale bar: (A–F) = 20 μm, (G and H) = 10 μm.

are consistent with *CSN5* acting in R cells to regulate R1-R6 targeting.

R Cell Differentiation and Pattern Formation Are Normal in *CSN5[1]*, *CSN5[2]*, and *CSN5[3]* Alleles

The targeting defect in *CSN5[1]*, *CSN5[2]*, and *CSN5[3]* does not result from transformation of R1-R6 neurons into R7 and/or R8 neurons that normally project into the medulla. The mistargeted neurons continue to express the R1-R6-specific markers *Rh1-LacZ* (Figure 2B) and *Ro-τlacZ* (Figure 1B). In the developing eye, Bar (i.e., expressed specifically in R1 and R6 neurons) (Figure 3B), Prospero (i.e., expressed in R7 neurons and non-neuronal cone cells) (Figure 3D), and Boss (Figure 3F) were expressed in patterns indistinguishable from wild-type. These results are consistent with plastic sections of homozygous adult mutant patches (Figure 3H). Of some 704 ommatidia scored from 10 independent mosaic patches, the number, organization, and morphology of *CSN5[1]* mutant R cells in some 697 ommatidia were indistinguishable from wild-type. In the remaining seven ommatidia, a single R cell was missing. Small numbers of missing R cells have been observed in other connectivity mutants (Garrity et al., 1996, 1999; Hing et al., 1999).

CSN5 Is Required in R Cells to Promote Lamina Glial Cell Migration

Developmental studies indicated that R1-R6 axons initially recognize lamina glia as intermediate targets in the developing lamina (Figure 4A) prior to making connections to specific lamina neurons several days later. Based on morphological studies, Steller and colleagues proposed that the establishment of precise patterns of R1-R6 projections relies on interactions between R1-R6 afferents and lamina glia cells (Perez and Steller, 1996). The notion that lamina glia, not lamina neuronal precursors, are intermediate targets for R1-R6 afferents was later supported by phenotypic analyses of *nonstop* and *hedgehog* mutants (Poeck et al., 2001). Defects in glial cell induction are not an indirect effect of R1-R6 mistargeting, as glial cells develop normally in other genetic backgrounds in which R1-R6 axons mistarget (i.e., *brakeless*; Rao et al., 2000; Senti et al., 2000).

To assess whether *CSN5* is required in R cells for the development of lamina glia, we analyzed target development using the glial-specific anti-repo antibody (Xiong et al., 1994), both in homozygous *CSN5[1]* and in genetic mosaics in which mutant R cell axons project into a wild-type brain. While R cell-dependent lamina precursor cell proliferation and neuronal differentiation occurred normally (Figures 5B and 5D), lamina glial cell development was disrupted in *CSN5[1]* homozygotes. This mutant phenotype also was observed in wild-type targets innervated by *CSN5[1]* mutant R cells (Figures 4B and 4E). In wild-type, three layers of glia (epithelial, marginal, and medulla glia) surround the lamina plexus where R1-R6 axons terminate. In *CSN5[1]* homozygotes and mosaic animals in which the eye is mutant and the target is wild-type, there is a marked reduction in lamina glial cell number (approximately 46% of that in wild-type; see Figure 4C), and the remaining cells form disorganized rows.

The effects of *CSN5[1]* on lamina epithelial and marginal glia were compared to the effects on medulla glia by analyzing mutant animals carrying enhancer trap markers for these two populations. The lamina glial cells were visualized using 1.3D2 enhancer trap line (Figure 6A), and the medulla glial cells with MZ97 (Figure 6C). The number of marginal and epithelial cells was markedly reduced, and the cells were highly disorganized (Figure 6B). In contrast, there was no appreciable effect on the number of medulla glia in *CSN5[1]* mutants. While the medulla glial cell layer was disrupted in some cases, in most preparations a continuous row of medulla glia formed (Figure 6D).

Defects in lamina glial cell development could be due to a defect in glial cell migration or differentiation. To distinguish between these two possibilities, we analyzed the distribution of glial cells in the developing optic lobe using anti-repo antibody as a marker. Lamina glial cells are derived from groups of cells flanking the lamina plexus; glial cells generated in these regions then migrate into the target. In wild-type, glial cells express Repo as they migrate into the R cell projection field. In *CSN5[1]*, there is an increase in the number of cells that

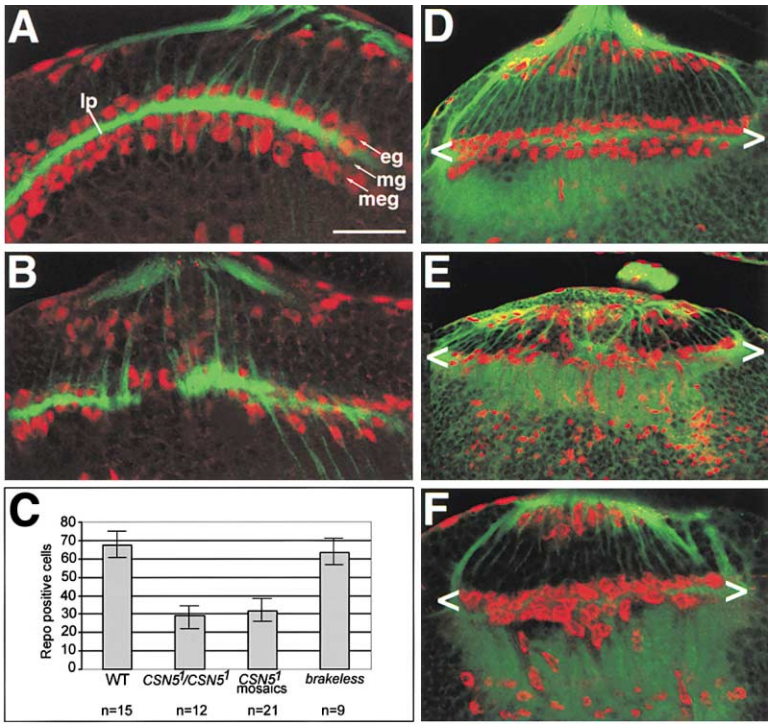

Figure 4. Lamina Glial Cell Development Requires *CSN5* Function in R Cell Afferents

(A and B) Glial cells ([eg] = epithelial, [mg] = marginal, [meg] = medulla) and R cell axons in the lamina were visualized with glial-specific anti-repo (red) antibody and mAb24B10 (green), respectively; (lp) = lamina plexus. Scale bar: 30 μm. (A) Wild-type. (B) In genetically mosaic animals in which *CSN5¹/CSN5¹* mutant R cells innervate a heterozygous target, glial cell number is reduced in a manner similar to *CSN5¹* homozygotes (see panel [C]).

(C) Quantification of glia in the target of indicated genotypes.

(D–F) Neurons and lamina glial cells were visualized with anti-HRP (green) and anti-repo (red) antibodies, respectively. (D) Wild-type. (E) *CSN51/CSN51* eye mosaic. (F) *brakeless/brakeless* (see Rao et al., 2000; Senti et al., 2000).

accumulate at the lateral edges (Figure 6F). In wild-type, there are 12.1 ± 2.0 cells (n = 17), whereas in homozygous *CSN5¹* mutants there are 21.9 ± 3.4 cells (n = 13) and in *CSN5¹* mutant eyes projecting into a wild-type target, there are 19.0 ± 3.1 cells (n = 24). This accumulation is consistent with a failure of many glial cells to migrate in from the margin and parallels the decrease in the number of marginal and epithelial glial cells in the lamina. Based on these observations, we conclude that lamina glial cell migration is disrupted in *CSN5¹* mutants.

These defects in lamina glial cell development and in R1-R6 targeting are similar to those observed in *nonstop* mutants (Poeck et al., 2001). In contrast to *CSN5*, *nonstop* is required in lamina glial cells, and not in R cell afferents, for glial cell differentiation. The R1-R6 hyperinnervation phenotype in *nonstop* is similar to that in *CSN5* mutants (Poeck et al., 2001; see Figure 1B). These data argue that *CSN5* functions in R cells to promote normal cell migration and development of lamina glial cells. It remains possible that *CSN5* plays a dual function in R cells being required in a non-cell-autonomous fashion to induce glial cell development and in a cell-autonomous function in R1-R6 growth cone targeting. We think this is unlikely, as R1-R6 mutant neurons in a small patch of mutant retinal tissue target to the lamina plexus in a fashion similar to wild-type, while mutant neurons show marked mistargeting when imbedded in large mutant patches (data not shown).

JAB1/CSN5 Is Expressed in the Optic Lobe Neuropil

The JAB1/CSN5 protein could act directly in R cell growth cones to mediate interaction with target cells or alternatively, it could act indirectly, for instance, in R cell nuclei to control gene expression required for R1-R6 targeting. To gain insight into how JAB1/CSN5 regulates R cell differentiation and interaction between R cell growth cones and lamina glial cell targets, the subcellular distribution of JAB1/CSN5 was determined. To assess the JAB1/CSN5 expression pattern in the developing visual system, a Myc-epitope tag was inserted into the C terminus of the *CSN5* open reading frame in a genomic construct (*myc-CSN5*) and introduced into flies. Four independently generated transgenic lines were analyzed. In each case, the tagged transgene rescued the *CSN5* lethality and connectivity phenotypes. This supports the view that the expression pattern observed using a Myc-tagged genomic construct accurately reflects the endogenous expression of JAB1/CSN5. Third instar eye-brain complexes of animals carrying the transgene were stained with anti-Myc antibody. As shown in Figure 7A, anti-Myc staining is predominantly localized to the cytoplasm throughout the developing eye disc. In the developing optic ganglia, anti-Myc staining is prominent in the lamina plexus (Figure 7B), which at this stage in development largely comprises R cell axons and growth cones. Myc immunoreactivity also was enriched in the medulla neuropil. This is the region to which the R7 and R8 cells, as well as lamina and medulla neurons, send their axons. The expression of the Myc-tagged genomic construct in each of four transgenic lines was identical. The JAB1/CSN5 expression pattern is similar to other proteins previously shown to be required for signaling in growth cones (e.g., Dock and Pak) during R cell axon guidance and targeting (Garrity et al., 1996, 1999; Hing et al., 1999).

CSN5/JAB1 Is Required for R Cell Differentiation

As the *CSN5¹* homozygous phenotype was indistinguishable from the *CSN5¹* over a deficiency, it seemed likely that this allele was a strong loss-of-function or a

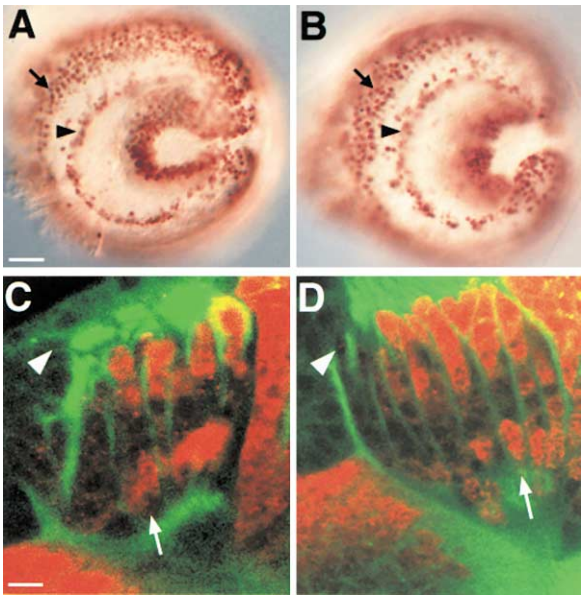

Figure 5. Lamina Neuronal Development Is Normal in *CSN5*^{1}

(A and B) BrdU incorporation marks the three proliferation zones in the developing optic lobe: the outer proliferation center ([OPC], indicated with arrows), the lamina precursor cells ([LPC], indicated with arrowheads), and the inner proliferation center (IPC). Neuroblasts in the OPC give rise to the LPC. R cell innervation drives the final cell division of LPC. (A) Wild-type. (B) In *CSN5*^{1}/*CSN5*^{1}, the proliferation patterns of LPC and other zones were indistinguishable from those of wild-type.

(C and D) Differentiating lamina neurons and R cell afferents were visualized with anti-Elav (red) and anti-HRP (green) antibodies, respectively. R cell innervation induces the differentiation of LPC (indicated by arrowheads) into lamina neurons. (C) Wild-type. (D) In *CSN5*^{1}/*CSN5*^{1}, the columns of Elav-expressing cells (see arrow) are normally organized.

Scale bar: (A and B) = 10 μm, (C and D) = 5 μm.

null allele. Nevertheless, as all three *CSN5* alleles are missense mutations, we set out to identify mutations that delete *CSN5* coding sequence to unambiguously establish the null phenotype. Protein null mutations were generated by imprecise excision of a weak *CSN5* mutant that carries a P element within the promotor region of the *CSN5* locus (see Experimental Procedures). Six *CSN5* protein null alleles were isolated. In contrast to the pupal lethality observed in animals carrying *CSN5* missense mutations (i.e., *CSN5*^{1}, as well as *CSN5*^{2} and *CSN5*^{3}) or the late larval and pupal lethality of previously described partial loss-of-function mutations in *CSN5* (Freilich et al., 1999), protein null homozygotes die in early larval stages. A heat shock-driven *CSN5* cDNA transgene rescues the lethality (see Experimental Procedures). One null allele, designated *CSN5*^{N} (Figure 8A), was chosen for further phenotypic analysis. It is the result of a deletion of the entire open reading frame as demonstrated through both PCR analysis and Southern blots (data not shown).

As *CSN5*^{N} larvae do not survive to third instar, the eye phenotype was assessed in genetically mosaic animals. *CSN5*^{N} eye tissue was generated using FLP recombinase expressed under the control of the eyeless promoter (ey-FLP) to promote eye-specific FRT-mediated mitotic recombination between wild-type and *CSN5*^{N} chromosomes. Under these conditions, more than 50% of the cells within the eye disc are homozygous mutant (C.-H. Lee and S.L.Z., unpublished data). Plastic sections of adult mutant eye tissue stained with toluidine blue revealed that the position of R cells and their morphology were abnormal (Figure 8B). Abnormalities in differentiating R cells were observed in *CSN5*^{N} clones in developing third instar eye discs (Figures 8C and 8D). Expression of Elav, an early neuron-specific nuclear protein, and Chaoptin, a later appearing R cell surface protein, is greatly reduced in the mutant clones. However, Futsch, a neuronal microtubule-associated protein that appears at an early stage of R cell differentiation, and Boss, an early R8-specific protein, were expressed normally (Figure 8C). Expression of other markers including Dachshund, Cubitus interruptus (Ci), Delta, and Atonal was similar to wild-type (Figure 8C; data not shown). Cell cycle progression in the eye disc appeared normal as assessed using BrdU incorporation (Figure 8C). The lack of a cell cycle phenotype as assessed with BrdU incorporation is consistent with the observation that the size of homozygous *CSN5*^{N} mutant clones was similar to sister clones homozygous for the wild-type allele (i.e., "twin spot"; data not shown).

These data indicate that JAB1/CSN5 is critical for neuronal differentiation, while earlier fate specification, patterning, and cell cycle events appear to proceed largely as in wild-type. Cone cell development was also disrupted in *CSN5*^{N} as the expression of the cone cell markers, Cut and Sparkling, was reduced (data not shown). As R cells induce cone cells (Flores et al., 2000), it is possible that cone cell defects are due to an indirect effect of loss of JAB1/CSN5 in R cells.

The COP9 Signalosome Is Required for R Cell Differentiation

In both plant and animal cells, JAB1/CSN5 assembles into a complex called the COP9 signalosome (Kwok et al., 1998; Freilich et al., 1999). While the function of this complex in animal cells is not understood, in plant cells, though highly pleiotropic, it plays a crucial role in regulating light-dependent gene expression. To address whether the *CSN5* null phenotype reflects the loss of function of the COP9 signalosome function, we assessed the role of another subunit, CSN4, in eye development. We acquired a *CSN4*-null mutant strain that harbors a deletion of part of the *CSN4* coding region (S. Rencus and D.S., unpublished data). A detailed characterization of *CSN4*^{N} will be described elsewhere. In *Arabidopsis*, all reported subunit mutations lead to a loss of the COP9 signalosome (Karniol and Chamovitz, 2000). Similarly, in both *CSN4*^{N} and *CSN5*^{N} fly mutants, an intact COP9 signalosome does not form (data not shown). As shown in Figures 8B and 8C, *CSN4*^{N} clones in the eye contain R cells with markedly abnormal cellular morphologies similar to R cells in *CSN5*^{N} mutant clones. *CSN4*^{N} and *CSN5*^{N} mutant phenotypes in developing eye discs are also similar, with a marked reduction of Elav and Chaoptin expression (Figures 8C and 8D). These data are consistent with the *CSN5* null mutant phenotype reflecting the loss of the COP9 signalosome function in the developing eye.

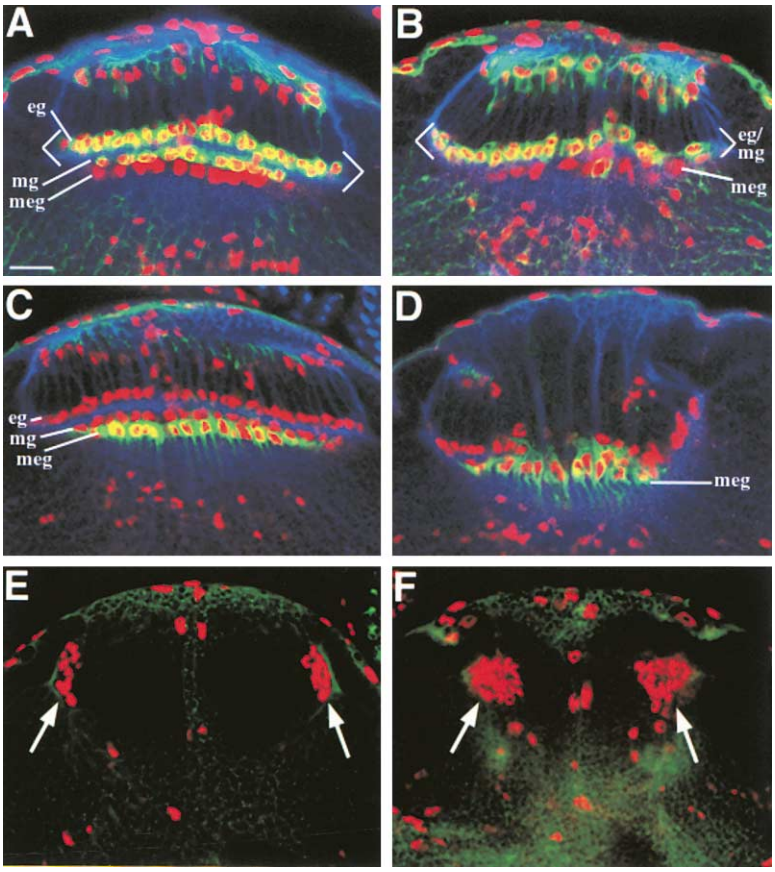

Figure 6. Lamina Glial Cell Migration Is Disrupted in *CSN5*^{1} Mutants

(A) In wild-type, the 1.3D2 Gal 4 transgene drives β−galactosidase expression (green) in marginal (mg) and epithelial (eg) glial cells, but not in medulla glial cells (meg). (B) In *CSN5*^{1} mutants, this marker is expressed in fewer cells and they are no longer organized into two discrete rows. (A and B) A pan-glial marker, anti-repo, is shown in red.

(C) In wild-type, the MZ97 marker (green) is expressed in the meg cells, but not in the mg or eg cells (D). The medulla glial cell number and organization are largely unaffected in *CSN5*^{1} mutants. (C and D) Anti-repo staining is shown in red.

(E and F) In wild-type, epithelial and marginal glial cells migrate into the lamina from the dorsal and ventral edges (arrows). Here, glial cells contact R cell axons which induce their migration into the lamina target. In *CSN5*^{1} mutant mosaic animals in which R cells are mutant (but the glial cells are heterozygous), the glial cells accumulate at the edges as they do not migrate into the target area (see text for quantification). Anti-Repo staining is shown in red.

Scale bar: (A–F) = 40 μm.

Discussion

R cell axons engage in a complex dialogue with developing lamina cells. R cell growth cones produce Hedgehog and Spitz, which induce the final cell division of lamina neuronal precursors and the differentiation of lamina neurons, respectively. R cell axons have also been proposed to produce a third signal that induces lamina glial cell development, although the molecular identity of this signal remains unknown. Lamina glial cells, but not lamina neurons, are essential for R1-R6 targeting, as genetic ablation of lamina glial cells results in R1-R6 mistargeting to the medulla. In this paper, we demonstrate that JAB1/CSN5 protein is required for the development of lamina glial cells and is enriched in regions in the optic lobe neuropil containing R cell growth cones. As these glial cells act as intermediate targets in the lamina, the R1-R6 neurons mistarget to the medulla. In *JAB1/CSN5* mutants, lamina glial cells accumulate at the lateral edges of the developing lamina neuropil where they contact R cell growth cones. The accumulation of lamina glia at these sites and the genetic requirement in R cells are consistent with the conclusion that JAB1/CSN5 is required for signaling between R cell growth cones and lamina glial cells.

JAB1/CSN5 Is Required in R Cell Afferents to Induce Lamina Glial Cell Development

Does JAB1/CSN5 play a direct role in regulating intercellular signaling pathways in R cell growth cones necessary for the induction of lamina glial cells? Recent studies in mammalian cells provide a precedent for JAB1/CSN5 function in contact-dependent intercellular interactions. JAB1/CSN5 was shown to physically interact with LFA1, an integrin critical for the interaction between T cell receptors and antigen-presenting cells (Bianchi et al., 2000). Interaction between the T cell receptor and the MHC-peptide complex is converted into a high-affinity complex through the recruitment of additional adhesion molecules linking the cells together. This includes LFA1, an integrin that directly interacts with I-CAM, an Ig domain-containing protein expressed on the surface of the antigen-presenting cell. The costimulatory interaction between T cell and antigen-presenting cells creates a specialized contact area termed an "immunological synapse." Transport and clustering of signaling components at the interface between these two cells leads to further amplification of intercellular signaling mediating the communication between them. It is attractive to envision that JAB1/CSN5 may contribute to the construction and/or maintenance of a structure supporting reciprocal interactions between the lamina glial cells and R cell axons.

As the JAB1/CSN5 protein is also localized to R cell bodies, it is possible that it regulates lamina development indirectly, for instance, by controlling the expression of signals necessary for lamina glial cell induction. Indeed, studies in mammalian cells in culture and in plant cells provide a precedent for JAB1/CSN5 function in transcriptional regulation (reviewed in Chamovitz and

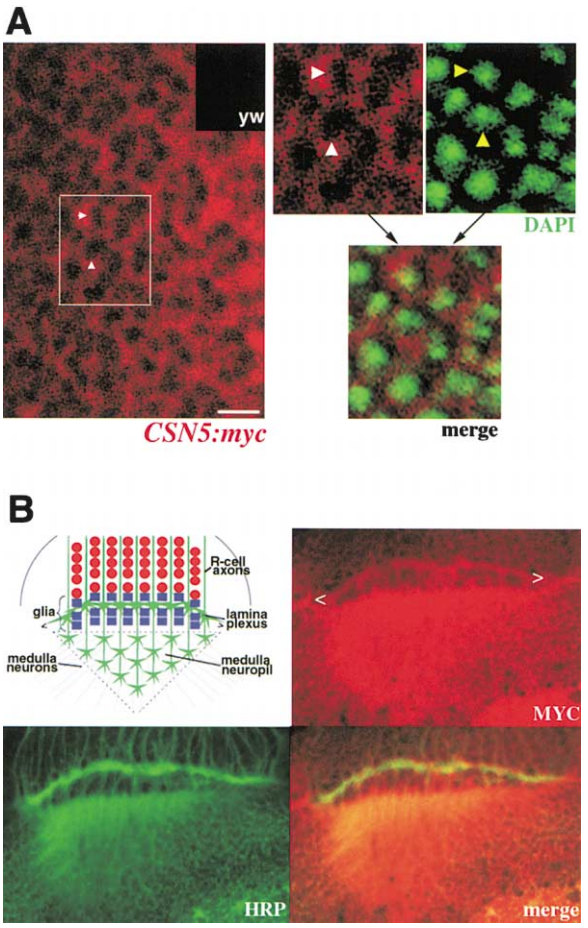

Figure 7. The JAB1/CSN5 Protein Is Enriched in the Cytoplasm of Cells in the Eye Disc and in the Optic Lobe Neuropil

A *CSN5* genomic fragment tagged with a Myc-epitope rescued the mutant phenotype (see text). Anti-Myc antibody staining was then used to assess the distribution of JAB1/CSN5 expressed in the eye and optic lobe.

(A) JAB1/CSN5 is broadly expressed in the cytoplasm of cells (anti-Myc antibody) in the developing eye disc. Little overlap in anti-Myc (red) and a nuclear marker, DAPI (green) staining, was observed (arrowheads). No detectable staining was seen in eye discs from larvae lacking the transgene (inset, yw), indicating that anti-Myc antibody is specific to JAB1/CSN5:Myc. Scale bar = 10 μm.

(B) JAB1/CSN5 expression in the developing optic lobe was visualized with anti-Myc antibody (red) and compared to anti-HRP antibody, which selectively stains neuronal cell surfaces and is highly enriched on axons and growth cones in the developing lamina and medulla neuropils. The schematic diagram shows that innervating R cell axons pass through the optic stalk (OS) and fan out in the optic lobe. R1-R6 axons stop in the lamina where they elaborate large growth cones between the epithelial and marginal glial cells that merge to form the lamina plexus. R7 and R8 axons pass through the lamina and terminate in the medulla neuropil. At this stage in development, the lamina plexus is largely comprised of R cell axons and growth cones. Both medulla and lamina neurons also send axons into the medulla neuropil. JAB1/CSN5 staining is enriched in both the lamina and medulla neuropils.

Segal, 2001). Overexpression of mammalian JAB1/CSN5 stimulated Jun-dependent transcriptional activation (Claret et al., 1996), and the binding of JAB1/CSN5 to diverse proteins affects AP1 mediated transcription. Further support for the notion that JAB1/CSN5 regulates AP1-dependent transcription came from studies in which the level of the COP9 signalosome, including CSN5, was elevated in cells by overexpressing another COP9 signalosome component, CSN2 (Naumann et al., 1999). These data raise the intriguing possibility that JAB1/CSN5 and the COP9 signalosome (more generally) regulate Jun-dependent intracellular pathways. However, as *Jun* mutations in the fly eye do not disrupt R1-R6 targeting (data not shown), it is unlikely that the R cell innervation and glial cell induction defects reflect disruption of Jun regulation. Therefore, JAB1/CSN5 must be impinging on other signaling pathways to regulate R1-R6 targeting. Identifying these targets is an important future goal.

A role for JAB1/CSN5 in regulating gene expression is consistent with studies in *Arabidopsis*. Like true loss-of-function mutations in other subunits of the COP9 signalosome, antisense RNA inactivation of *CSN5* in *Arabidopsis* derepresses a light-dependent developmental pathway in the dark (Kwok et al., 1998). The COP9 signalosome facilitates the accumulation of the COP1 protein in the nucleus (reviewed in Osterlund et al., 1999). COP1, probably acting as an E3 ubiquitin-ligase, in turn promotes degradation of the light-dependent transcriptional activator HY5 (Osterlund et al., 2000). Hence, in the dark, the COP9 signalosome inhibits HY5-dependent transcription by regulating the cellular levels of HY5 itself. Through an unknown mechanism, light inactivates the COP9 signalosome function, export of COP1 from the nucleus, and stabilization of HY5. These findings raise the intriguing possibility that signaling events at the R cell growth cone could lead to changes in gene expression necessary for interactions between the growth cone and glia cell targets that are dependent upon the COP9 signalosome.

The COP9 Signalosome Is Required for R Cell Differentiation

A role for JAB1/CSN5 in photoreceptor cell differentiation was revealed through analysis of protein null alleles. Cells within null mutant clones express reduced levels of two neuronal differentiation markers: Elav, an RNA binding protein required for neuronal differentiation and Chaoptin, an R cell-specific, cell surface protein. Another neuronal marker, Futsch (a neuron-specific, microtubule-associated protein) is expressed normally. Consistent with a role for JAB1/CSN5 in differentiation, null mutant R cells survive into the adult and exhibit disrupted cellular morphologies. Interestingly, R cell neurons lacking Elav exhibit similar morphological defects (Campos et al., 1985; Homyk et al., 1985). Defects are not restricted to R cell differentiation, however, as the expression of two cone cell homeodomain proteins, Cut and Sparkling, were also markedly reduced in *CSN5* null mutants. As R cells induce cone cell development, it remains unclear whether this reflects a role for CSN5 in cone cells. Early eye patterning genes such as *Cubitis interruptus*, *dachshund,* and *atonal* are expressed normally. Hence, JAB1/CSN5 plays a crucial role in R cell and cone differentiation but is largely dispensable for early patterning in the eye disc.

As JAB1/CSN5 is found in *Arabidopsis* and *Drosophila* in two forms (a multisubunit complex, the COP9 signalo-

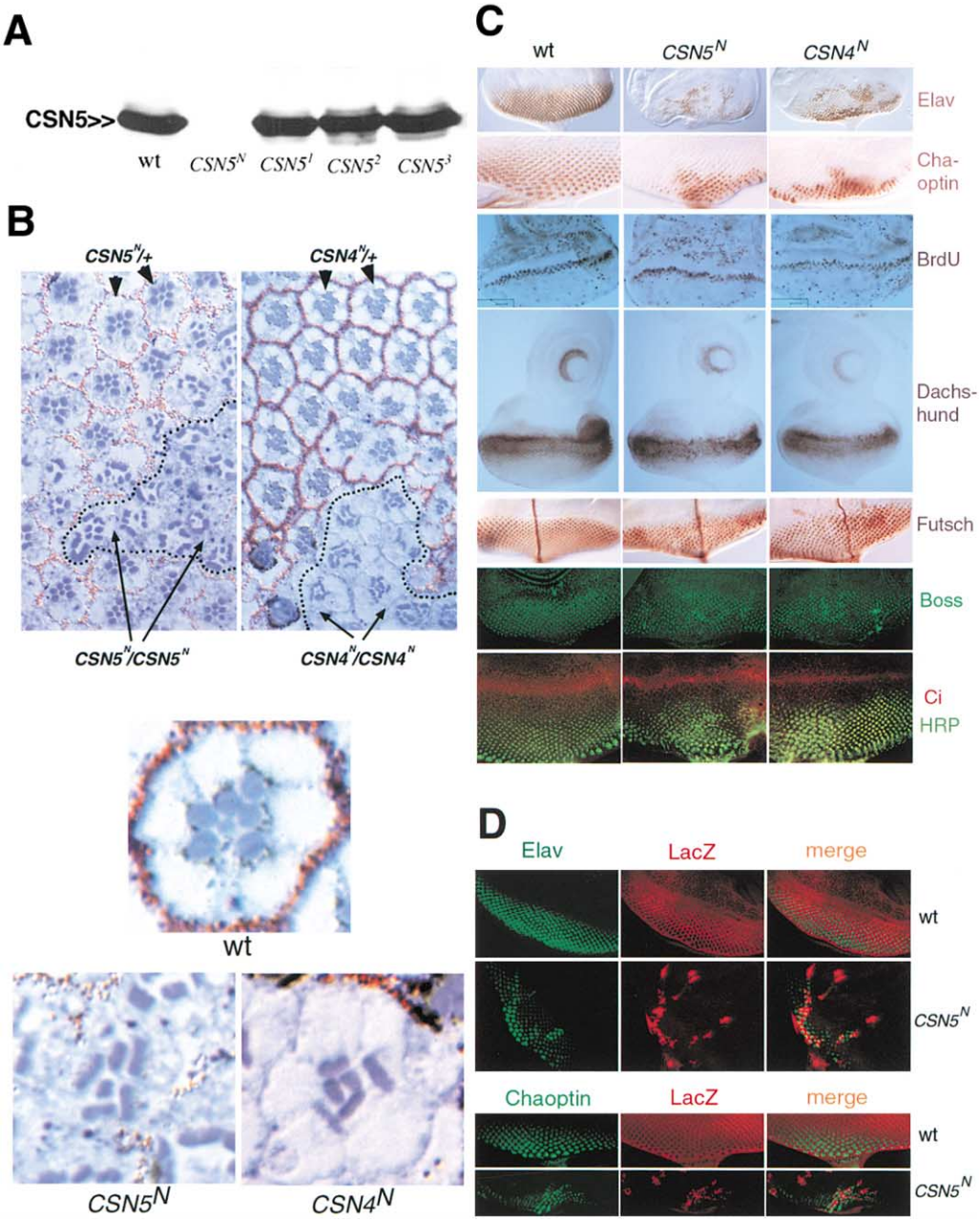

Figure 8. *CSN5*^{N} and *CSN4*^{N} Mutant Eye Phenotypes Are Similar

(A) A Western blot of *CSN5* mutant and wild-type extracts probed with anti-JAB1/CSN5 antibody, as indicated: *CSN5*^{1}, *CSN5*^{2}, and *CSN5*^{3} are missense mutations; *CSN5*^{N} is a deletion mutant.

(B) Plastic sections of *CSN5*^{N} and *CSN4*^{N} mutant patches in the adult eye. Homozygous mutant tissues were generated by FRT-mediated mitotic recombination. Sections were stained with toluidine blue. The morphology of R cells was highly disrupted in the mutant patches, which are outlined with dotted lines. Three lower panels show magnified views of individual ommatidia.

(C) In both *CSN5*^{N} and *CSN4*^{N} mutant eye discs, generated by ey-FLP-induced mitotic recombination (genotype: ey-FLP;FRT(82B)*CSN5*^{1}/FRT(82B) *l(3R)E2F* or *M(3)*; see Experimental Procedures), expression of Elav and Chaoptin was greatly reduced. The expression of other markers was similar to wild-type. A cell-autonomous marker to identify the clone was not used here but was used for the analysis of *CSN5*^{N} mutant clones shown in (D). In control experiments, greater than 50% of the cells in the eye disc were homozygous mutant.

(D) *CSN5*^{N} mutant clones were generated using ey-FLP-mediated recombination in conjunction with a cell-autonomous marker on the wild-type chromosome (Armadillo-LacZ). Wild-type cells, red; *CSN5*^{N} mutant cells, not red (i.e., unstained); Elav and Chaoptin, green. Homozygous mutant cells show a marked reduction in Elav and Chaoptin expression.

some, and in a smaller complex-independent form) (Freilich et al., 1999; Kwok et al., 1998), defects in eye development in *CSN5* mutants may reflect the function of the JAB1/CSN5 monomer or the COP9 signalosome. To address this issue, we analyzed the role of another component of the COP9 signalosome, *CSN4*, in eye de-

velopment. In *Drosophila CSN4* mutants, the CSN complex does not form, but the JAB1/CSN5 monomer remains (E.O. and D.A.C., unpublished data). As *CSN4* and *CSN5* null phenotypes are indistinguishable, we conclude that they reflect the essential function of the COP9 signalosome in eye development.

Missense Mutations in the JAB1/MPN Domain Selectively Disrupt Interactions between R Cells and Lamina Glial Cells

As opposed to the general R cell differentiation defects caused by the *CSN4* and *CSN5* null mutations, three different *CSN5* missense mutations isolated in two independent screens specifically disrupt interactions between R cell afferents and lamina glial cells but do not lead to defects in R cell differentiation. These mutations are clustered within the JAB/MPN domain and lead to nonconservative substitutions in amino acids shared between plant, fly, and human JAB1/CSN5.

What could be the biochemical basis for this specific connectivity phenotype? JAB1/CSN5 may be required for a single function; a reduction in its activity in the missense mutations may disrupt only the most sensitive process (e.g., lamina glial cell induction). Alternatively, JAB1/CSN5 may serve multiple functions. The missense mutations may selectively disrupt a subset of them that are dependent upon specific residues within the JAB/MPN domain, while the null mutation abolishes all JAB1/CSN5 functions. It is conceivable that these different functions are inherent to the different cellular forms of JAB1/CSN5 (e.g., complexed versus noncomplexed forms). In *Arabidopsis*, the CSN5 in the COP9 signalosome is primarily nuclear, while the CSN5 monomer is primarily cytoplasmic (Kwok et al., 1998). Therefore, the cytoplasmic localization of JAB1/CSN5 reported here could suggest that noncomplexed forms of JAB1/CSN5 are involved in R cell growth cone glial cell interactions, similar to the cytoplasmic form of JAB1/CSN5 that interacts with LFA1 (Bianchi et al., 2000).

Recent studies suggest that the multiple roles attributed to JAB1/CSN5 and the COP9 signalosome may reflect their function in regulating protein degradation. The COP9 signalosome positively regulates SCF complexes through the removal of Ned8 modifications from the cullin subunits (Lyapina et al., 2001), allowing for E3 activity. Mutations in the COP9 signalsome lead to the accumulation of multiple neddylated cullins in fission yeast (Zhou et al., 2001). Mutations in the *Arabidopsis* COP9 signalosome lead to the accumulation of ubiquitinated proteins (Peng et al., 2001). While the exact role of the COP9 signalosome in these processes is still obscure, we propose that the function of the COP9 signalosome and JAB1/CSN5 in R cells involves the regulation of protein degradation, probably through its interaction with specific E3 ligases. The specific missense mutations in $CSN5^1$, $CSN5^2$, and $CSN5^3$ may selectively disrupt the interactions with a subset of neddylated substrates involved in controlling interactions between R cell growth cones and lamina glial cells. Further biochemical and genetic analyses are required to determine the relationship between the structure of JAB1/CSN5 and its function in R1-R6 targeting, glial cell induction, and R cell differentiation.

Experimental Procedures

Genetics and Rescue

$CSN5^1$ was isolated from the histological screen. $CSN5^2$ and $CSN5^3$ alleles were obtained from a lethal mutant collection in 89C/D (Sanchez-Herrero et al., 1985). $CSN5^P$ and $CSN4^P$ alleles were obtained from the Berkeley *Drosophila* Genome Project (BDGP). $CSN5^N$ and $CSN4^N$ deletions were created by mobilizing the P elements inserted in these loci. The sequences surrounding the $CSN5^N$ and $CSN4^N$ deletions were amplified by polymerase chain reaction and sequenced. In $CSN5^N$, the entire transcription unit is deleted. In $CSN4^N$, a 200 bp region from ~50 bp upsteam of the open reading frame start and extending downstream is deleted. The *CSN5* locus was mapped to the cytological location, 89C/D, by its failure to complement *Df(3R)Sbd104* and *Df(3R)RK6-3*, obtained from Ian Duncan (Hopmann et al., 1995). Several genomic DNA fragments from the region defined by the proximal break of *Df(3R)RK6-3* and the distal break of *Df(3R)Sbd104*, a cosmid clone carrying an ~30 kb genomic DNA, and *CSN5* cDNAs under heat shock promotor were cloned and transformed into flies as described (Spradling and Rubin, 1982). Heat shock rescue of lethality was performed by mating w;$CSN5^N$/TM3 to w;P[w+,hs-*CSN5*] line 2/+; *Df(3R)RK6-3*/TM3. The transgene was followed by eye color. While the cross was maintained at 25°C, heat shocks were at 37°C for 45 min every 12 hr. Rescue of lethality was assessed by comparing white eye and red/orange eye progeny of each genotype raised with and without heat shock. The genotypes of adult progeny with red/orange eye color (with the transgene) were as follows: (with heat shock) $CSN5^N$/TM3, 19 (35%); *Df(3R)RK6-3*/TM3, 20 (36%); $CSN5^N$/*Df(3R)RK6-3*, 16 (29%); and (without heat shock) $CSN5^N$/TM3, 16 (55%); *Df(3R)RK6-3*/TM3, 13 (45%); $CSN5^N$/*Df(3R)RK6-3*, 0 (0%). The genotype of progeny with white eye color (without the transgene) were as follows: (with heat shock) $CSN5^N$/TM3, 27 (53%); *Df(3R)RK6-3*/TM3, 24 (47%); $CSN5^N$/*Df(3R)RK6-3*, 0 (0%); and (without heat shock) $CSN5^N$/TM3, 43 (55%); *Df(3R)RK6-3*/TM3, 35 (45%); $CSN5^N$/*Df(3R)RK6-3*, 0 (0%). Four independent transgenes P[w+,hs-*CSN5*] all behaved similarly. Rescue of the R cell axon projection in the third instar larva was performed by mating P[w+,hs-*CSN5*] line 1/Y; *Df(3R)RK6-3*/TM6B males to $CSN5^N$/TM6B females and comparing the projection pattern between male and female progeny.

Genetic Mosaic Analysis

Genetic mosaic clones were created using X-ray-induced (Ashburner, 1989) and ey-FLP-mediated FRT mitotic recombination (Xu and Rubin, 1993; Newsome et al., 2000b). Near whole-eye homozygous mutant clones of $CSN5^1$, $CSN5^N$, and $CSN4^N$ were generated by ey-FLP-mediated FRT recombination in *trans* to a chromosome carrying either a *Minute* or a cell lethal, and LacZ transgene expressed under a ubiquitous promotor. The fraction of the mutant tissues created was estimated by absence of anti-LacZ staining. To assess R1-R6 targeting phenotype, $CSN5^1$ locus was recombined onto *Rh1-LacZ* transgene, a marker specific for R1-R6 axons, and w;$CSN5^1$ *Rh1-LacZ*/p(w+)90E first instar larva were x-irradiated to induce single mutant patches. Cryostat sections were prepared, mosaic patches were photographed to document their location, and sections were stained with anti-LacZ antibody to visualize R1-R6 targeting. To assess the adult eye phenotype of $CSN5^1$, $CSN5^N$, and $CSN4^N$, both X-ray irradiation and ey-FLP-mediated FRT recombination were used. Tangential eye sections were performed as described (Van Vactor et al., 1991).

Histology

Whole-mount immunohistochemistry, cryostat section, and BrdU labeling protocols were essentially as described in Garrity et al. (1996). The preparation of toluidine blue-stained semithin sections (1 μm and 2 μm) of adult mosaic heads for light microscopy was as described in Salecker and Boeckh (1995). A mouse anti-Myc antisera (Developmental Studies Hybridoma Bank) (1:25) was used for *myc-CSN5* immunolocalization. R cell projections were visualized with mAb24B10 (1:200) and secondary HRP-conjugated goat anti-mouse (1:200) antibodies. Lamina glia were stained using a rat anti-repo antibody (1:100). R cells were stained with mAb24B10, mAb22C10 (1:25), and a mouse anti-Elav (1:25) antibody. Subsets

of R cells were visualized with rabbit anti-Bar (1:100), mouse anti-Prospero (1:100), and mouse anti-Boss (1:2000) antibodies. For confocal laser scanning microscopy (Bio-Rad), preparations were incubated with secondary goat anti-rabbit or mouse IgG coupled to Cy3 (1:400) or FITC (1:200) (Jackson Immunoresearch).

Molecular Biology

Overlapping genomic P1 clones encompassing 89C7–89D1 region were isolated from the P1 library (Pierce and Sternberg, 1992). Modified P1 clones with two ends of the original insert were made by digesting the original P1 insert with BamH1 (the P1 vector does not have the restriction site) and religating back with the remaining ends. Each end was isolated by digesting the modified P1 clones with the BamH1/Not-1 or BamH1/Sfi1 and was labeled using the DIG labeling system (Boehringer Mannheim) for in situ hybridization to polytene chromosomes. According to the in situ results, the proximal *Df(3R)RK6-3* and the distal *Df(3R)Sbd104* breaks were contained with P1 06002 and P1 06297 clones, respectively. Genomic Southern blots were performed to physically map the *Df(3R)RK6-3* and *Df(3R)Sbd104* breaks using ^{32}P-labeled 5.5 kb BamH1 and 12 kb BamH1/Sau3A P1 genomic fragments as probes. Genomic rescue constructs were built by subcloning genomic fragments into pCaSpeR4 transformation vectors. *CSN5* cDNA clone was isolated from a 0–24 hr embryo cDNA library in EXLX(+) (Palazzolo et al., 1990). UAS-*CSN5* and hs-*CSN5* were constructed by subcloning *CSN5* cDNA into the UAS and heat shock transformation vectors.

Biochemistry

Western blot analysis was performed as described in Hing et al. (1999).

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