

Decoding Cilia Function: Defining Specialized Genes Required for Compartmentalized Cilia Biogenesis

Tomer Avidor-Reiss,¹ Andreia M. Maer,²
Edmund Koundakjian,¹ Andrey Polyakov,¹
Thomas Keil,⁴ Shankar Subramaniam,^{2,3}
and Charles S. Zuker^{1,*}

¹Howard Hughes Medical Institute and Division of
Biological Sciences and Department of
Neurosciences

²San Diego Supercomputer Center

³Departments of Bioengineering and Chemistry
and Biochemistry

University of California at San Diego
La Jolla, California 92093

⁴Max Planck Institute of Biochemistry
Martinsried
Germany

Summary

The evolution of the ancestral eukaryotic flagellum is an example of a cellular organelle that became dispensable in some modern eukaryotes while remaining an essential motile and sensory apparatus in others. To help define the repertoire of specialized proteins needed for the formation and function of cilia, we used comparative genomics to analyze the genomes of organisms with prototypical cilia, modified cilia, or no cilia and identified ~200 genes that are absent in the genomes of nonciliated eukaryotes but are conserved in ciliated organisms. Importantly, over 80% of the known ancestral proteins involved in cilia function are included in this small collection. Using *Drosophila* as a model system, we then characterized a novel family of proteins (OSEGs: outer segment) essential for ciliogenesis. We show that oseg genes encode components of a specialized transport pathway unique to the cilia compartment and are related to prototypical intracellular transport proteins.

Introduction

Cilia are microtubule-rich, hair-like cellular extensions that perform essential motile and sensory functions. In sperm and in unicellular eukaryotes, a motile form of cilia called flagellum propels cells to their destination, while in epithelial cells, multiple motile cilia beat synchronously to stir extracellular fluid (Sleigh, 1974). In vertebrate photoreceptor cells and invertebrate mechano- and chemoreceptor neurons, the entire sensory transduction machinery is housed in a specialized cellular compartment derived from the cilium (Bloodgood, 1990; Sleigh, 1974). This domain, known as the outer segment, is a hallmark of these sensory neurons and

an outstanding example of subcellular compartmentalization as a strategy to optimize function (Figure 1A).

Given the wide range of cells and tissues that contain cilia, and the extraordinary diversity of roles performed by cilia (Bloodgood, 1990; Rosenbaum and Witman, 2002; Sleigh, 1974), a basic question in cell biology is how ciliogenesis is orchestrated and to what extent common mechanisms underlie this process. Cilia formation begins when the basal body, a centriole-related structure, serves as a template for the assembly of the axoneme. This process can proceed through two different mechanisms. In most motile and sensory cilia, the basal body docks to the plasma membrane, and a bud-like structure containing the axoneme and the ciliary membrane projects out from the cell body; since the ciliary membrane and the axoneme are assembled concurrently as a compartment separated from the cell body, we refer to this process as compartmentalized ciliogenesis. In a few cases, however, such as in the sperm cells of *Drosophila* (Tokuyasu et al., 1972) and the flagella of the parasite *Plasmodium* (Sinden et al., 1976), the entire axoneme is first assembled inside the cytosol and only later is either extruded or matures into a flagellum (i.e., cytosolic biogenesis).

Unlike cytosolic biogenesis, the process of compartmentalized ciliogenesis requires that cilia, flagella, and outer segments transport their building blocks—proteins and metabolites—from the cell soma. Genetic and biochemical studies in the biflagellated green alga *Chlamydomonas* have singled out kinesin II, dynein 1b, and 17 additional proteins named *intraflagellar transport* (IFT) particle proteins as candidate proteins involved in flagella biogenesis. IFT particle proteins are proposed to function as macromolecular rafts traveling up and down the flagellum, via kinesin and dynein, transporting axonemal precursor proteins to their growing tips (Rosenbaum and Witman, 2002). Consistent with this postulate, mutations in the *Chlamydomonas* IFT particle proteins IFT88 and IFT52 produce very short flagella (Brazelton et al., 2001; Pazour et al., 2000). Similar results are seen in *C. elegans* mutants defective in the IFT orthologs OSM-5 and OSM-6 (Perkins et al., 1986).

We are interested in the biogenesis of cilia, with particular emphasis on the formation of sensory outer segments and developed a novel bioinformatics approach to identify genes involved in ciliogenesis. Our strategy is based on the hypothesis that the ancestral eukaryote was a ciliated unicellular organism (Cavalier-Smith, 2002), and that cilia and flagella were independently lost throughout evolution from several eukaryotic groups (Cavalier-Smith, 2002). By comparing the genomes of ciliated and nonciliated organisms, we have identified a collection of candidate genes important for cilia formation and function. In addition, by phylogenetically examining orthologs in organisms with “compartmentalized” versus “cytosolic” axonemes, we isolated a large subgroup selectively expressed in *Drosophila* sensory outer segments, but not in sperm, and characterized a novel family of proteins (OSEGs: outer segment) essential for compartmentalized ciliogenesis. Together, these stud-

*Correspondence: charles@flyeye.ucsd.edu

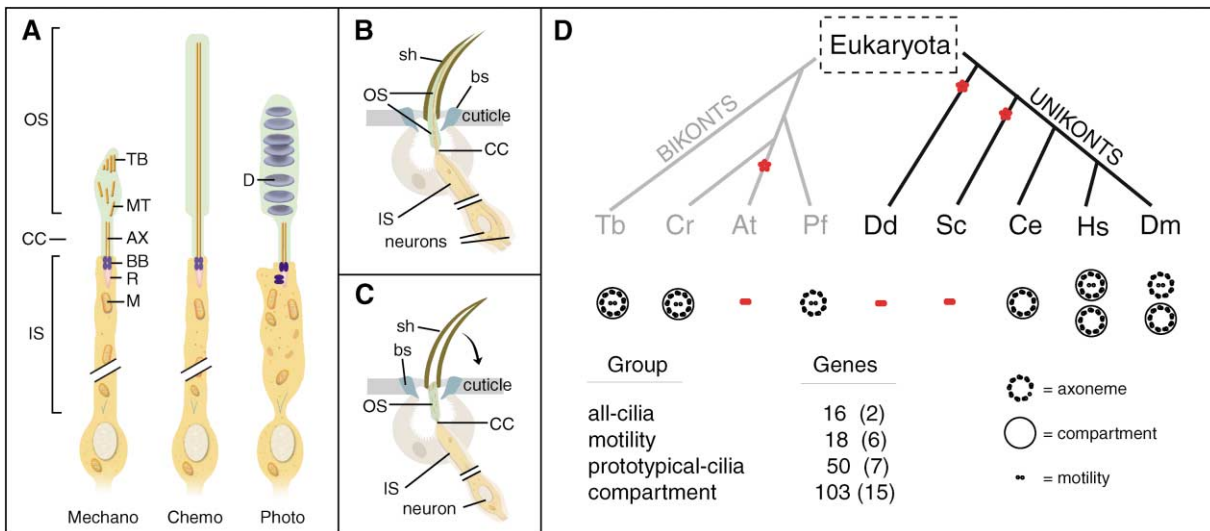


Figure 1. Sensory Cilium Structure and Evolution

(A) The sensory cilium is composed of a connecting cilium (CC) and the outer segment (OS). Cilia are anchored to the inner segment (IS) through the basal body (BB) and rootlet (R). M, mitochondria; AX, axoneme; MT, microtubules; D, discs; TB, tubular body; this is a rich array of tightly packed microtubules at the tip of the cilia. Diagram of *Drosophila* external chemosensory (B) and mechanosensory (C) organs, bristle socket (bs) and bristle shaft (sh).

(D) Ciliary genes are selectively lost from nonciliated organisms but maintained in ciliated organisms. Eukaryote phylogeny showing the two main lineages: Bikonts (gray) and Unikonts (Stechmann and Cavalier-Smith, 2002, 2003). The ancestral nature of the eukaryotic cilia is evident by its presence in organisms from both lineages: *D. melanogaster* (Dm), *H. sapiens* (Hs), *T. brucei* (Tb), and *C. reinhardtii* (Cr). In three independent events (indicated by red stars), cilia were lost in lineages leading to *A. thaliana* (At), *D. discoideum* (Dd), and *S. cerevisiae* (Sc). Similarly, compartmentalized cilia were lost in *P. falciparum* (Pf), while motile cilia were lost in *C. elegans* (Ce). Shown below the phylogenetic tree is an illustration of the various ciliary structures present in the different organisms used in the bioinformatics searches. Also shown is a summary of the screens, which provides the number of genes identified in each search (the numbers in parenthesis refer to previously known genes); Supplemental Figure S1 online shows a flowchart illustrating the four bioinformatics screens.

ies establish a compelling bioinformatics strategy to help decode gene function and lay the foundation for a comprehensive dissection of eukaryotic ciliogenesis and outer segment development.

Results and Discussion

Ciliary Genes Are Selectively Conserved in Ciliated Organisms

In order to identify specialized genes essential for cilia biogenesis and function, we undertook a phylogenetic screen that identified genes conserved in the genomes of ciliated organisms but absent in nonciliated eukaryotes. We reasoned that gene loss can be used as a powerful tactic to map gene function, particularly if the biological process in question (e.g., cilia biogenesis in this case) is conserved in distantly related species and if it was lost more than once during evolution. We chose eight species representing the two major lineages of eukaryotic evolution (Stechmann and Cavalier-Smith, 2002, 2003), and which included nodes where cilia were lost or modified during the evolution of eukaryotes (Figure 1D).

Because *Drosophila* contains experimentally tractable motile and sensory cilia and has an extensively annotated genome, we selected it as the anchor for these studies. We performed BLAST searches against the proteome of five ciliated (*H. sapiens* [Hs], *C. elegans* [Ce], *P. falciparum* [Pf], *C. reinhardtii* [Cr], and *T. brucei* [Tb]) and three nonciliated organisms (*A. thaliana* [At], *S. cere-*

visiae [Sc], *D. discoideum* [Dd]) and searched for orthologs of the ~14,000 *Drosophila* genes in each of these species using a “reciprocal best hit” algorithm (Li et al., 2003; Remm et al., 2001). Because the *T. brucei* and *C. reinhardtii* genomes are incomplete (see Experimental Procedures), we considered a ciliary protein as conserved in Bikonts if it was present in either of those two species (Cr/Tb). Similarly, a partial draft of the *Dictyostelium discoideum* (Dd) proteome is now available; this organism displays exquisite motility, yet it lacks ciliated structures, thus providing a robust bioinformatics counterscreen.

Because all ciliated organisms have an axoneme but may differ in their mode of ciliogenesis, or whether they have motile or nonmotile cilia, we suspected that distinct sets of proteins might be required during biogenesis of the various forms of cilia. Therefore, we applied our screening strategy to four different search routines (see Supplemental Figure S1 at <http://www.cell.com/cgi/content/full/117/4/527/DC1>): First, to identify genes involved in processes common to all cilia, like axoneme formation, we compared all ciliated versus all nonciliated eukaryotes (i.e., genes conserved in Hs, Dm, Pf, Cr/Tb, and Ce but not in At, Sc, or Dd). Second, to identify genes involved in cilia motility (either of compartmentalized or cytosolic origin), we compared organisms with motile cilia versus those with nonmotile or no-cilia (i.e., genes conserved in Hs, Dm, Pf, and Cr/Tb but not in Ce, At, Sc, or Dd). Third, to identify genes involved in cilia compartmentalization, we compared organisms

with compartmentalized cilia biogenesis versus cytosolic biogenesis (e.g., genes conserved in Hs, Dm, Ce, and Cr/Tb but not in Pf, At, Sc, or Dd). Finally, to identify genes that may be unique to organisms that have both motile and compartmentalized cilia, we also searched for genes shared between *Drosophila* and organisms with prototypical cilia (i.e., Hs and Cr/Tb but not in Ce, Pf, At, Sc, or Dd).

From a total of 121,243 predicted transcriptional units and 141,000 ESTs (we used ESTs in *Chlamydomonas* due to the lack of an assembled partial proteome), we identified a total of 187 ancestral genes (Supplemental Table S1): (1) 16 conserved in all ciliated organisms, but absent in nonciliated (all-cilia subset); (2) 18 present only in organisms with motile cilia (motility subset); (3) 103 common only to organisms with compartmentalized cilia biogenesis (compartment subset); and (4) 50 shared only between organisms with prototypical cilia (both motile and compartmentalized; prototypical-cilia subset).

To evaluate the performance of the screen, we asked whether known genes implicated in ciliogenesis are indeed enriched in this collection. A search of the literature revealed that there are 36 genes that have been implicated in ciliogenesis in either flies or in other organisms and were part of the likely ancestral repertoire of genes in the primitive eukaryotic cell (e.g., conserved in organisms from both ancestral eukaryotic lineages; see Experimental Procedures). This set includes specialized genes whose primary role is in cilia biogenesis and function (e.g., dynein arms, IFTs), as well as genes that may also participate in other cellular processes (e.g., dynein light chains). A breakdown of these genes using the criteria utilized in our screen is shown in Figures 1D and 2. Remarkably, 30 out of the 36 known genes (>80%) are included in the 187 ancestral gene collection obtained in our bioinformatics screen; of the remaining six, five also function outside the cilia and were filtered out because they are present in nonciliated organisms (four dynein subunits and myosin VIIA), and one (left/right-dynein) was eliminated because it did not have an ortholog in Tb or Cr.

The selectivity of the screen is also illustrated by examining the genes in the motility subset (Figure 2): all six known ciliary genes recovered in this collection, in fact, encode proteins involved in motility (four axonemal dynein subunits, a radial spoke protein, and Mbo2, a protein important for flagella waveform). In addition, of the remaining 12 candidate motility genes in this subgroup (Supplemental Table S1), five are specifically expressed in testis (see Figure 2), a tissue highly enriched in motile cells. Taken together, these results substantiate the logic of the approach and our search criteria.

The Compartment Gene Set

We are particularly interested in the formation of sensory outer segments, therefore we focused on the genes in the cilia-compartment subset both as a platform for gene discovery and for dissecting mechanisms of outer segment biogenesis. Curation of the 103 candidates in this group (Figure 2 and Supplemental Table S1 online) suggested that several may not have a direct role in ciliogenesis, yet they cosegregated with our selection criteria. These included ion channels, signal-transduc-

tion components, transcription factors, and metabolic enzymes. In order to extract “ciliary” genes from this subset, we demanded that candidates meet two additional search criteria. First, many genes involved in sensory cilia formation share an upstream regulatory sequence known as the X box, often at 150 to 50 nucleotides upstream from the translation start site (Dubruielle et al., 2002; Swoboda et al., 2000). A general search of the *D. melanogaster* and *C. elegans* genomes for the presence of the 14 nucleotides consensus X box motif demonstrated that this sequence is much too abundant to be used as a primary screen (for instance, 2449 of Dm and 1897 of Ce genes contain such a motif); however, as a secondary screen, it selected 41 candidates from the cilia-compartment subset. Notably, over 90% of the known ciliary genes in the compartment subset (14/15) are included in these 41 genes. Second, compartmentalized cilia in *Drosophila* are found only in chemo- and mechanosensory neurons. Because these neurons are scattered all over the fly body and comprise a minute fraction of the fly cells, available EST databases contain none, or very few, representative ESTs (data not shown). Based on this premise, we searched the compartment subset for genes that contained 0–4 ESTs and identified 48 candidates. Importantly, these 48 candidates contain nearly all of the known ciliary genes in the original collection (13/15). Together, these two secondary screens identified a total of 30 genes that overlapped both the X box and EST filters (see Figure 2 and Supplemental Table S1 online); these were chosen for biological validation.

Ciliary Compartment Genes Are Expressed in *Drosophila* Sensory Neurons

Genes involved in compartmentalized ciliogenesis should satisfy two important requirements. First, the genes should be expressed in ciliated sensory cells. Second, the proteins must be essential for outer segment formation or function.

The genes selected in the compartment subset encode members of several protein groups, including IFT proteins, Bardet-Biedl syndrome (BBS)-related polypeptides, C2 domain-containing proteins, small G proteins, a group of “coil-coil” proteins, and a family of six WD-domain proteins (OSEGs). Below we provide a short summary of these families.

BBS is a heterogeneous genetic disorder that is characterized by retinal dystrophy, renal malformation, learning disabilities, and obesity. Six BBS genes have been cloned, and several were recently implicated in ciliogenesis (Ansley et al., 2003). *Drosophila* has three BBS orthologs, and all three were selected in this screen (BBS1 and BBS8 as part of the compartment subset, and BBS4 as part of the prototypical-cilia subset). Interestingly, this collection also includes two additional proteins sharing a similar domain organization (CG5142 and CG4525; Figure 2), which we suggest encode new BBS members.

The C2 domain is a ~120 amino acid sequence that functions as a Ca²⁺-dependent membrane-targeting module in proteins involved in signal transduction (e.g., protein kinase C, cytosolic phospholipase A2) or transport processes (e.g., synaptotagmin I, rabphilin) (Shao et

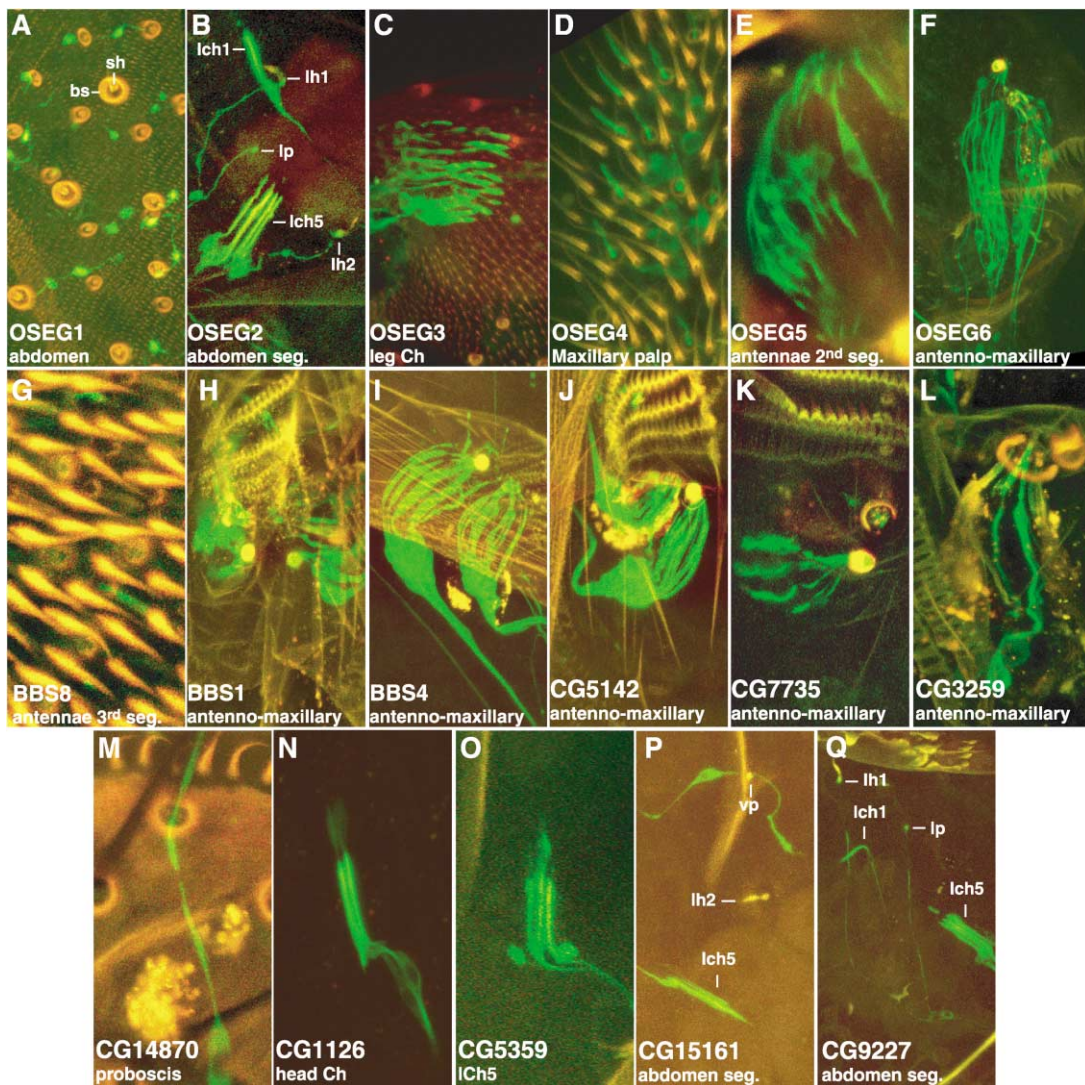


Figure 3. Ciliary Compartment Genes Are Selectively Expressed in Ciliated Sensory Neurons

Upstream regulatory regions of candidate ciliary compartment genes were fused to Gal4 and expressed in animals carrying UAS-GFP or UAS-GFP- α 1tub84B reporters; *oseg1* (A); *oseg2* (B); *oseg3* (C); *oseg4* (D); *oseg5* (E); *oseg6* (F); BBS8 (G); BBS1 (H); BBS4 (I); CG5142 (J); CG7735 (K); CG3259 (L); CG14870 (M); CG1126 (N); CG5359 (O); CG15161 (P); CG9227 (Q). lch, lateral chordotonal organs; lh, lateral hairs; lc, lateral campaniform organ. See Table 1 for a compilation of expression data.

transgenic flies expressing Gal4 promoter fusions. Individual lines were crossed to flies containing UAS reporters and examined for GFP expression in larvae and adult animals.

In *Drosophila*, there are three types of ciliated cells: sperm, mechanosensory, and chemosensory neurons. Mechanosensory and chemosensory cilia are assembled through compartmentalized ciliogenesis, while the sperm tail is assembled via cytoplasmic ciliogenesis. Figure 3 and Table 1 demonstrate refined specificity in the anatomical sites of expression of all 17 genes: each transgene is restricted to ciliated cells, with BBS4 and the 15 candidate compartment genes expressed exclusively in neurons of mechanosensory and chemosensory organs. The remaining one, *Tctex2/LC2* (a dynein light chain subunit from dynein arms and cytosolic dyneins), was also expressed in sperm cells (data not

shown; see Patel-King et al. [1997]). No other sites of expression were observed for any of the transgenes. Taken together, these results strongly authenticate our bioinformatics strategy, provide a new perspective into the evolution of cilia, and set the foundation for a comprehensive use of this approach in other biological processes.

oseg Genes Are Specifically Required for Outer Segment Formation

To gain insights into the biology of outer segment biogenesis, we next screened for mutants defective in candidate cilia-compartment genes. *Drosophila* mutants with outer segment defects are expected to be mechanosensory defective; we therefore screened mutagenized F3 lines for the presence of mechanosensitive flies (Han et al., 2003; Kernan et al., 1994) and recorded

Table 1. Expression Pattern of Ciliary Compartment Genes

	Larvae			Adult		Adult
	A-M	Ch	ES	Ch	ES	T
<i>oseg1</i>	✓	✓	✓	✓	✓	—
<i>oseg2</i>	✓	✓	✓	✓	✓	—
<i>oseg3</i>	✓	✓	—	✓	✓	—
<i>oseg4</i>	✓	✓	✓	✓	✓	—
<i>oseg5</i>	—	✓	—	✓	✓	—
<i>oseg6</i>	✓	✓	ND	✓	✓	—
CG7735	✓	✓	ND	✓	✓	—
CG9227	✓	✓	✓	✓	✓	—
CG14870	—	—	—	—	✓	—
CG5142	✓	✓	✓	✓	✓	—
CG1126	✓	✓	—	—	✓	—
CG3259	✓	✓	—	✓	✓	—
CG15161	✓	✓	✓	✓	✓	—
CG14825	✓	✓	✓	✓	✓	—
CG13691	✓	✓	✓	—	✓	—
CG13232	✓	✓	✓	✓	✓	—
CG5359	✓	✓	✓	—	✓	✓

ND: not determined.

Flies expressing promoter fusions for all 17 candidate genes were examined for anatomical sites of GFP expression in larvae and adult animals (see also Figure 3). There was no expression of the reporter in nonciliated cells. A-M, larval anteno-maxillary complex; Ch, chordotonal organs; ES, external sensory organs; T, testis.

mechanoreceptor currents (MRC; Figure 5A) and trans-epithelial potentials (TEP; Figure 5B) from candidate lines. Mutations that affect the cilia are predicted to show defective MRC (Walker et al., 2000). In contrast, mutations that affect the function or development of the support and accessory cells should abolish both the MRC and the TEP (Barolo et al., 2000). We recorded MRCs and TEPs from multiple bristles in various uncoordinated lines and selected complementation groups with normal TEP but defective MRC and tested them in chemosensory and sperm motility assays. Two complementation groups with abnormal mechano- and chemosensory responses but normal sperm motility mapped near the location of *oseg1* and *oseg2*, respectively (Figures 5A–5C). We expected that mutant alleles would carry missense or nonsense mutations, and that introduction of the wild-type gene into mutant animals should rescue their behavioral and physiological phenotype. Indeed, *oseg1*¹⁷⁹ and *oseg1*¹⁰ alleles had stop codons in *oseg1* (Figure 4A), and the *oseg2* allele contained a nonconservative substitution in the *oseg2* gene (Figure 4B). More importantly, introduction of the wild-type *oseg1* and *oseg2* genes by germline transformation rescued the uncoordinated and MRC defects of *oseg1* and *oseg2* mutants (Figure 4C and data not shown).

To analyze the phenotype of *oseg1* and *oseg2* mutants in detail, we examined the ultra-structure of the sensory cilium by EM serial section analyses. Wild-type mechano- and chemosensory dendrites contain a striated rootlet, two basal bodies, a connecting cilium, and the outer segment (Figures 5D and 5H). *oseg1* and *oseg2* mutants have normal inner segments and an intact rootlet, basal bodies, and connecting cilium (Figures 5E–5F). However, both mutants display dramatic defects in outer segment morphology (Figures 5I and 5J): In mechanoreceptor neurons, *oseg1* has a striking reduction of the

distal-most end of the outer segment (the dendritic tip and tubular body), while *oseg2* has a total loss of the tubular body. In chemoreceptors, *oseg1* and *oseg2* both show severely shortened outer segments (data not shown). Together, these results firmly implicate *oseg* genes in ciliogenesis, and outer segment formation.

OSEGs Are Required for Selective Intraciliary Transport

HMM analyses and secondary structure predictions indicate that OSEGs are related to α - and β '-coatomers (Figure 4D), two proteins involved in intracellular trafficking (Kirchhausen, 2000). Significantly, clathrin heavy chain (Chc) also displays prominent domain similarity to OSEG family members (Figure 4D). Because outer segments (and cilia) are separated from the rest of the cell by a connecting cilium, they need to import their proteins from the cell soma and therefore might be expected to require specialized machinery to assemble a functional compartment.

If the OSEG proteins were essential for the transport of selective macromolecules into ciliary compartments, we would expect them to meet several criteria. First, in contrast to structural or signal transduction components of the outer segment, OSEGs should travel in and out of the outer segment, while concentrating primarily at the base of the cilia. This region of the cell is considered the cilium's "hub," a strategic place between the cell soma and the outer segment, and is hypothesized to function as the site where molecules targeted to the cilium are loaded and transported via the microtubule-based motors (Cole et al., 1998; Dwyer et al., 2001; Marszalek et al., 2000; Pazour et al., 1999). Second, ciliary cargo should be transported normally from the cell soma to the cilia base of *oseg* mutants, but it should be unable to enter the cilia and therefore may accumulate near the cilia base.

To examine the subcellular localization of OSEG proteins, we engineered translational fusions between all six OSEG family members and GFP. Each fusion protein was then targeted to ciliated sensory cells using a pan-neuronal promoter. In order to mark the position of the cilium, we co-labeled the cells with mab21A6 (Fujita et al., 1982), a monoclonal antibody that labels the base of the cilium at the inner/outer segment boundary. As predicted, all GFP-tagged OSEG proteins localize primarily at the base of the cilium (Figure 6) and can be found inside the sensory cilia (Figures 6G–6I).

To examine transport into outer segments, we needed to identify a candidate cargo protein, ideally one that requires either of the available mutants (*oseg1* or *oseg2*). Mechanosensory outer segments contain at their distal-most end a unique microtubule-rich structure known as the tubular body; this is the proposed site of channel anchoring and force generation in mechanosensory bristles (Figure 1A). The α -tubulin isoform in the tubular body is encoded by the α 1tub84B gene in *Drosophila*. Therefore, we hypothesized that α 1tub84B would be an ideal OSEG cargo. Figure 7A demonstrates that overexpression of a plain GFP reporter, or even a membrane tagged-GFP (data not shown), does not label the outer segment of ciliated neurons. However, if GFP is linked to α 1tub84B (i.e., a GFP- α 1tub84B fusion protein), it is

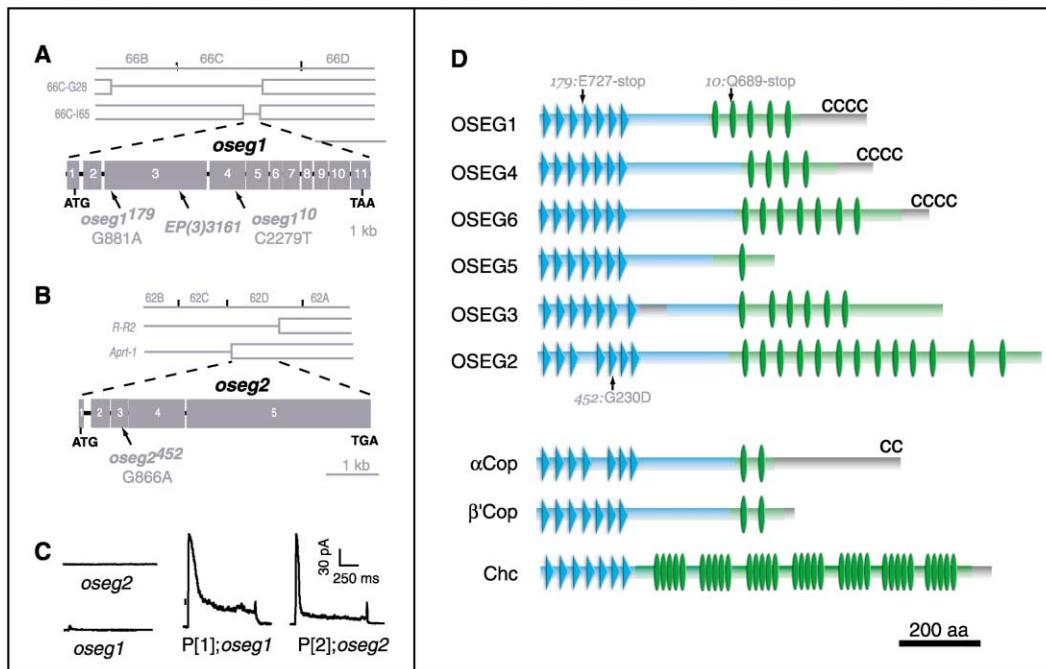


Figure 4. A Subgroup of Ciliary Compartment Genes, *osegs*, Encodes a Family of Proteins Related to Prototypical Intracellular Transport Proteins

(A and B) Genetic maps of *oseg1* and *oseg2* illustrating the chromosomal deficiencies used to localize both loci, the gene structures, and the nature of the mutations in the various alleles.

(C) *oseg1* and *oseg2* MRC defects are rescued by introduction of wild-type transgenes.

(D) Predicted structure and topology of OSEG family members (see also Supplemental Figure S1 online). Blue triangles, WD repeats; blue bar, β sheet rich domain; green ovals, TPR-like repeats; green bar, α helix rich domain; c, CxxC repeats. Arrows indicate the positions of the mutations.

now co-targeted with tubulin and functions as a robust reporter of $\alpha 1$ tub84B transport into the outer segment (Figure 7B).

Next, we introduced the GFP- $\alpha 1$ tubulin 84B reporter into *oseg* mutant backgrounds and examined its localization. Figures 7E–7G show that the GFP- $\alpha 1$ tub84B cargo completely fails to enter the outer segment of *oseg2* mutants, but is efficiently transported to the outer segments of controls and *oseg1* mutants. Furthermore, EM examination of *oseg2* mutant cells revealed a dramatic accumulation of microtubules at the base of the cilium (Figures 7K and 7M). These results prove that *oseg2*, but not *oseg1*, is essential for tubulin transport into the cilium and illustrate an important aspect of OSEGs function: OSEGs may play distinct roles, and different cargo are likely to be matched to specific OSEG members. Notably, the N-terminal WD domains of α -coatomers and clathrin have been implicated in cargo recognition and sorting (Eugster et al., 2000; ter Haar et al., 2000). The identification of six OSEG members with distinct N-terminal WD domains may provide the structural basis for selective cargo recognition within this family.

Our bioinformatics approach also identified two kinesin II subunits as cilia-compartment genes (see Figure 2). Kinesin II has been shown to be required for cilia assembly in a variety of organisms and was proposed to function as the anterograde motor carrying cargo from the base of the cilia to its distal tip (Cole et al.,

1998; Marszalek et al., 2000; Piperno and Mead, 1997). If OSEGs mediate the kinesin-based intraciliary transport, and if this transport were specifically required for outer segment formation, we reasoned that mutations in *klp64D*, the central component of *Drosophila* kinesin II (Ray et al., 1999), should generate *in vivo* phenotypes that resemble *oseg* defects. Thus, we generated flies defective in *klp64D* function and examined mechano- and chemosensory physiology and the transport and accumulation of $\alpha 1$ tub84B into sensory cilia. Figures 5 and 7 demonstrate that *klp64D* mutant animals share all of the hallmarks of *oseg2* mutants: (1) severe chemosensitivity, (2) a total loss of mechanoreceptor currents, (3) GFP- $\alpha 1$ tub84B completely failing to enter the outer segments, and (4) microtubules dramatically accumulating at the base of the cilium (Figure 7L). Furthermore, *klp64D* animals, just like *oseg2* mutants, have an almost complete loss of the tubular body, but have normal basal bodies and connecting cilia (Figures 5G and 5K); thus, kinesin II is also not essential for the assembly of the proximal ciliary structures, including axoneme components. Together, these results substantiate kinesin II as a critical player in OSEG function and validate the fundamental importance of intraciliary transport in outer segment (compartmentalized cilia) biogenesis.

Concluding Remarks

In this study, we used a novel bioinformatics screen relying on evolutionary gene conservation and gene loss

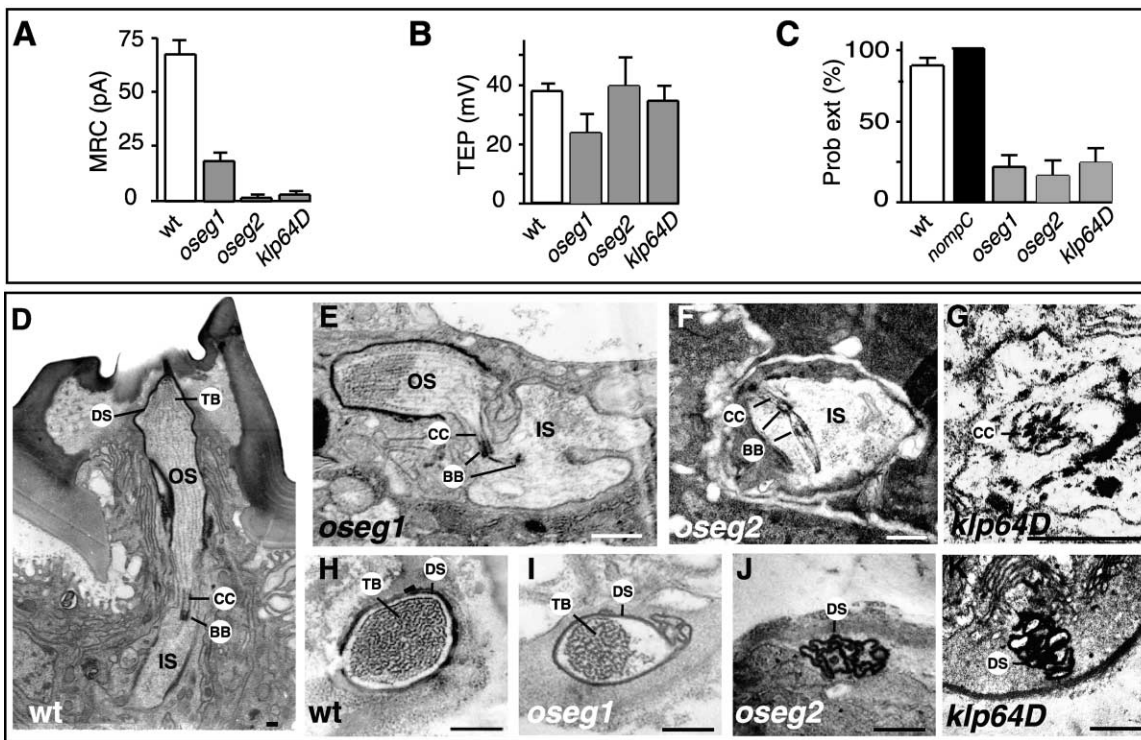


Figure 5. *oseg* Genes Are Required for Compartmentalized Cilia Formation and Function

oseg1, *oseg2*, and *klp64D* mutants have a dramatic loss of MRC (A) but have a robust TEP (B). They also have strongly attenuated chemosensory responses to sucrose (C), while responses of the mechanosensitive mutant *nompC* (Walker et al., 2000) are indistinguishable from controls (wt). Electron micrographs of wild-type (D and H), *oseg1* (E and I), *oseg2* (F and J), and *klp64D* (G and K) mechanoreceptor cells showing the IS, CC, OS, and TB. Note the severe reduction in microtubule content in *oseg1* (I) and the total loss of microtubules in the outer segment of *oseg2* (J) and *klp64D* (K), while the connecting cilium remains unaffected (E–G). Similar defects are seen in *oseg1* and *oseg2* chemosensory neurons (data not shown). DS, dendritic sheath; CC, connecting cilium; BB, basal body. Bar, 0.5 μ M.

as a paradigm to discover loci selectively involved in cilia formation and function. We showed that this strategy efficiently identified a wide spectrum of known ciliary proteins and dramatically enriched the repertoire of candidate ciliary genes. Because we focused on identifying ciliary genes of the ancestral eukaryotic cell (e.g., by selecting ciliary genes found in both Bikonts and Unikonts lineages), we did not expect to recover genes unique to specific lineages. However, by using selective combinations of genomes in our search algorithm, we were also able to define and distinguish between genes involved in cilia motility versus cilia compartmentalization; as additional genomes are completed, it should be possible to target new categories.

Approximately 200 genes (Table S1 on Cell website) were selected in the four searches described in this paper. We analyzed in detail the cilia-compartment subset and identified 27 genes as strong ciliary compartment candidates. We selected 15 for detailed in vivo expression studies and demonstrated that all were specifically expressed in compartmentalized cilia. Using a spectrum of curation strategies, we also examined many of the genes in the motility and prototypical-cilia subsets (see Experimental Procedures). Our analysis identified an additional collection of novel candidate ciliary genes (Figure 2). It will be of great interest to determine whether mutations in the human orthologs of these genes under-

lie cilia-based sensory, developmental, or reproductive disorders.

Ciliary genes that serve multiple cellular functions were not selected in this screen, mainly because they are still present in organisms that have lost ciliated structures. For example, dyneins are critical components of the ciliary motility apparatus, yet many were filtered out in our screens because they are also involved in intracellular transport in nonciliated organisms. Indeed, we suggest that the reason so few candidate genes were recovered in the “all ciliated organisms” subgroup is because proteins common to all cilia, like those involved in axoneme assembly, are also required in basic cellular processes and therefore conserved in nonciliated organisms (e.g., α -tubulin, β -tubulin, γ -tubulin, centrin, pericentrin, etc.).

What do cilia-compartment genes do? At a basic level, these genes should encode components of the intraciliary transport system and the cilia pore, a supramolecular structure that forms the gate into the cilia (Rosenbaum and Witman, 2002). Indeed, our screen identified all of the known IFT homologs found in *Drosophila*, including novel OSEG members. By extension, we suggest that the compartment group also contains the molecular components of the cilia pore complex.

Using a genetic screen relying on uncoordinated behavior and electrophysiological recordings of sensory

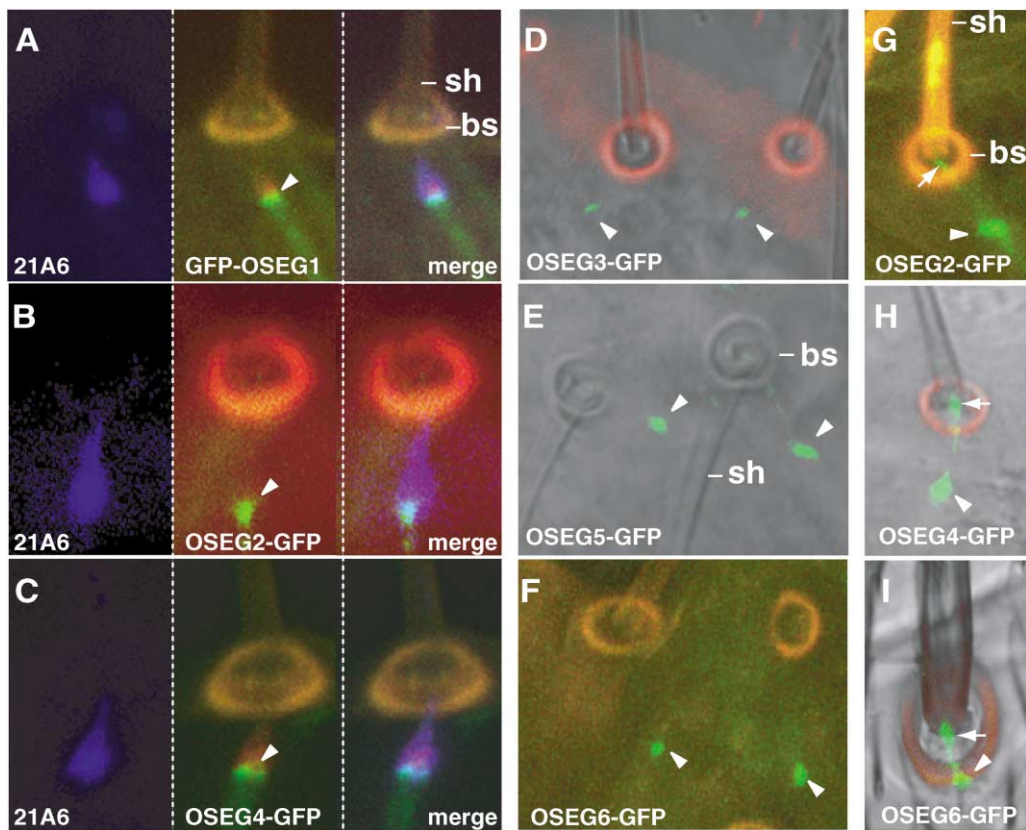


Figure 6. *osegs* Are Primarily Localize at the Base of the Cilia

(A–F) Confocal imaging of chemosensory organs expressing GFP-OSEG1 (A), OSEG2-GFP (B), OSEG4-GFP (B), OSEG3-GFP (C), OSEG5-GFP (E), and OSEG6-GFP (F) protein fusions. (A–C) To unequivocally identify the subcellular location of the OSEG-GFP fusions, whole-mounts were co-stained with mab21A6, an antibody that selectively labels the base of the cilium. Left panels show mab21A6 (blue), middle panels, OSEG-GFPs (green), right panels, merged images.

(G–I) OSEG-GFP labeling inside the outer segment of chemosensory organs (G and H) and mechanosensory organs (I). Orange labeling highlights the bristle socket (bs) and shaft (sh).

bristles, we isolated and characterized mutations in two *oseg* family members. We showed that *oseg1* and *oseg2* have distinct roles in ciliogenesis, but neither *oseg1* nor *oseg2*, or even kinesin II, are required for formation of the connecting cilium. These results demonstrate that the assembly of outer segment is orchestrated independently of the connecting cilia (and its axoneme). It will be of great interest to determine which cilia-compartment genes have a role in the biogenesis of this structure.

OSEGs are characterized by the presence of two major protein-protein interaction domains, WD and TPR repeats, implicated in the assembly of multiprotein complexes. Significantly, the most closely related proteins outside of the family are α - and β '-coatomer, two cargo-carrying proteins intimately involved in intracellular trafficking (Kirchhausen, 2000). Furthermore, clathrin heavy chains display striking domain similarity to the OSEG family: an N terminus consisting of 7 WD repeats and a C terminus consisting of \sim 35 TPR-like repeats known as CHCR motifs (ter Haar et al., 2000; Ybe et al., 1999). Interestingly, coatomers and clathrin-mediated transport systems use small G proteins of the Arf subfamilies as regulators of the transport process. Notably, our screen also identified ARL3 and ARL6, two Arf-like proteins, as components of the ciliary compartment group,

with ARL6 expression restricted to mechano- and chemosensory neurons.

What do OSEGs do? The *Drosophila oseg2* gene shares significant similarity with a 20 amino acid tryptic peptide from *Chlamydomonas* IFT172 (Cole et al., 1998). IFTs were originally identified as a group of proteins enriched in the flagella of *Chlamydomonas* dynein-1b mutants and absent in the flagella of kinesin II mutants. Because anterograde transport is blocked in kinesin mutants, and retrograde transport is abolished in dynein mutants, IFT particle proteins were proposed to function as molecular rafts transporting cargo up and down the axoneme. Multiple lines of evidence strongly support the proposal that OSEGs function as ciliary transport proteins. First, OSEGs are specifically expressed in ciliated cells, and the proteins are selectively localized to the cilia and cilia base. Second, OSEGs share structural similarity to prototypical intracellular transport proteins (e.g., clathrin, COP1). Third, *oseg2* mutants have specific defects in intraciliary transport. Fourth, *Drosophila* OSEGs are required for compartmentalized ciliogenesis (sensory cilia) but not for cytosolic ciliogenesis (sperm tail). Finally, flies defective in *oseg2* have nearly the same phenotype as mutants defective in *klp64D*, the ciliary motor. While there is very limited available data on *oseg*

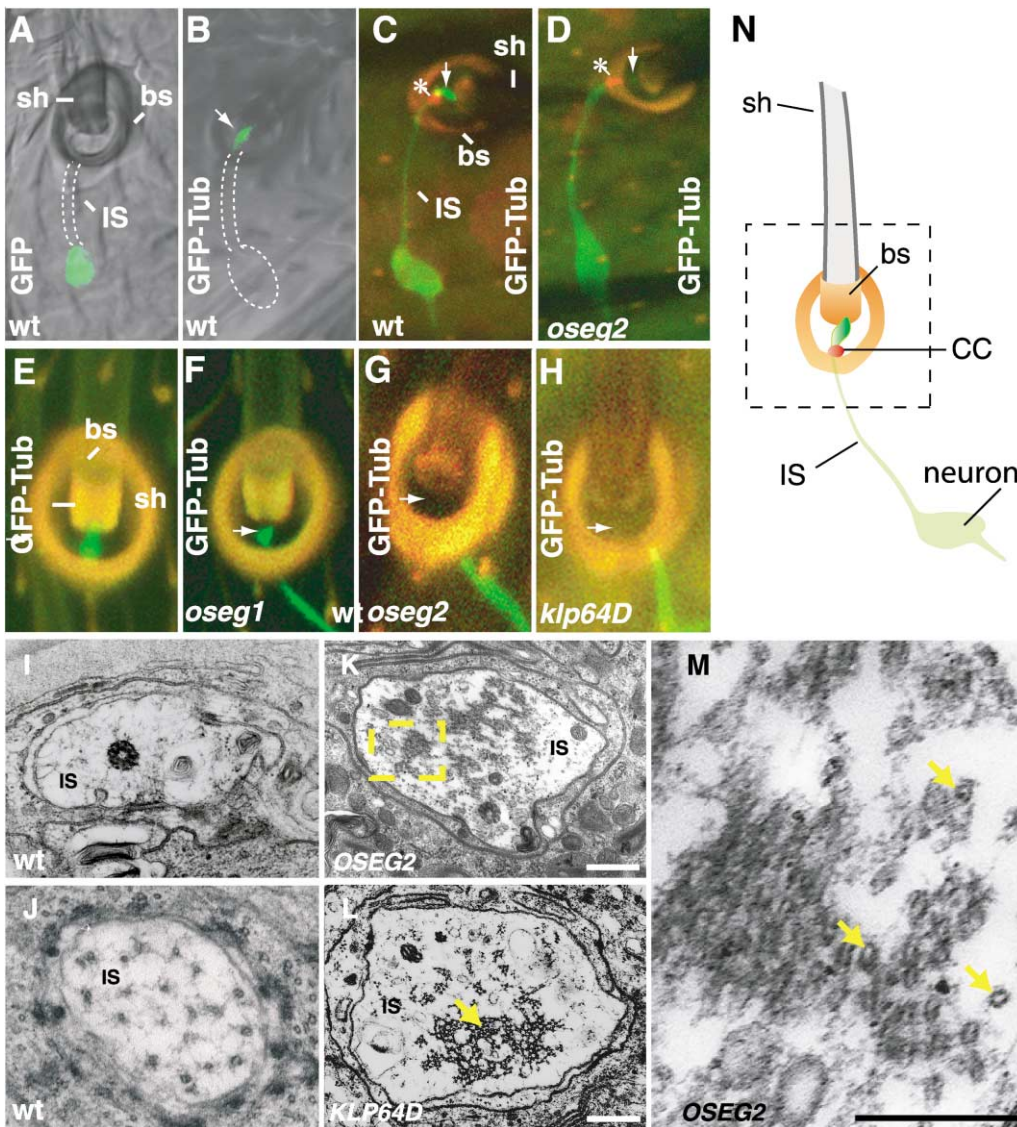


Figure 7. Outer Segment Cargo Is Mislocalized in *oseg2* and Kinesin II Mutants

(A and B) Confocal images of mechanoreceptor neurons expressing GFP (A) and GFP- α 1tub84B (B). Note selective labeling of outer segments by GFP- α 1tub84B (arrow). Dotted lines illustrate the location of the neurons and their projections.
(C–H) Confocal images of mechanoreceptors expressing GFP- α 1tub84B in wild-type (C and E), *oseg1* (F), *oseg2* (D and G), and *klp64D* (H) mutants. Note the mislocalization of GFP- α 1tub84B in *oseg2* and *klp64D*. (C) and (D) were also co-labeled with mab21A6 to mark the base of the cilium (red staining, star). Orange labeling highlights the bristle socket (bs) and shaft (sh).
(K–M) EM pictures demonstrating the dramatic accumulation of microtubules (yellow arrow and box) at the cilia base of *oseg2* (K and M) and *klp64D* (L); no microtubule accumulation is seen in the proximal part of the inner segment of mutant cells or in ciliated cells of wild-type controls (I and J).
(N) Diagram of a mechanosensory neuron labeled with GFP- α 1tub84B (green) and mab21A6 (red). The dashed box illustrates the approximate plane of the confocal images shown in (E)–(H); bristle socket (bs), shaft (sh), connecting cilium (CC).

orthologs in *Chlamydomonas*, several of the *oseg* orthologs in *C. elegans* genes map at, or near, the location of worm mutations leading to sensory cilia defects and implicated in cilia formation and maintenance (Perkins et al., 1986). For example, OSEG2 and OSEG5 are orthologs of OSM-1 (Signor et al., 1999) and CHE-2 (Fujiwara et al., 1999), and OSEG1 and OSEG3 are probably orthologs of DAF-10 and CHE-3 (Qin et al., 2001), respectively. Surprisingly, the integration of these proteins into a group of genes related to the main families of intracellular transport proteins had escaped notice. Our

results illustrate a common foundation in the organization of intracellular transport systems, whether mediating internalization of surface proteins, transferring cargo between organelles, or delivering components from the cell body to distal ciliary compartments.

Experimental Procedures

Bioinformatics

Genome sequence information used in this study was obtained in March 2003. These include *Drosophila* Genome Project, BDGP, (<http://www.fruitfly.org>), release 3 (17,878 protein sequences corre-

sponding to 13,759 genes) from UC Berkeley; WormBase (<http://www.wormbase.org>), release 100 (21,565 protein sequences); *T. brucei*, 15,300 protein sequences from Sanger Institute (http://www.sanger.ac.uk/Projects/T_brucei); The Hs (27,625 protein sequences); and At (27,242), Sc (6,333), Pf (5,300) protein sequences from the National Center for Biotechnology Information, NCBI, (<http://www.ncbi.nih.gov>). For the incomplete genome of Chlamydomonas, we used 141,000 sequence entries derived from EST, genomic, and cDNA sources and compared them using TBLASTN/BLASTX.

Bidirectional BLAST was used in the process of identifying orthologs and corresponding ESTs for *Drosophila* genes (<http://www.ncbi.nlm.nih.gov/BLAST>). After bidirectional BLASTP between two proteomes, an automatic clustering program, "Inparanoid," was used to identify the main orthologs (<http://www.cgb.ki.se/inparanoid>; Remm et al., 2001); only reciprocal best hits were considered as putative orthologs. Co-orthologs were identified by searching the OrthoMCL database (<http://www.cbil.upenn.edu/gene-family>; Li et al., 2003). A gene was considered an "ancestral candidate" if it was present in Unikonts (*Drosophila*) and Bikonts; the presence of a main ortholog or a co-ortholog in a nonciliated organism was sufficient to eliminate the candidate gene or move it from one subset to the other.

Putative X boxes (consensus: GTTGCCATGGCAAC) were identified in the 500 bp upstream of the initiator ATG from Dm and Ce genes using an in-house Perl program that scores pattern positional matches (A.M.M., unpublished data). Only putative X boxes that did not differ from the consensus by more than four nucleotides were accepted. ESTs for Dm genes (a total of 253,545 EST of which 30,384 were derived from testis) were identified using sequences from BDGP and Brian Oliver's testis EST project (Andrews et al., 2000) using reciprocal BLASTN. Only hits with at least 90% identity over the entire length of the EST were counted in our analysis.

Gene structures were predicted by FGENESH and validated by direct genomic and cDNA sequence analyses. Hidden Markov Model homology searches used MEME (Bailey and Elkan, 1994) and MAST (Karpplus et al., 1998). Domain analysis of OSEGs and α - and β '-coatomers proteins used SAM-T2 (<http://www.soe.ucsc.edu/research/compbio/HMM-apps/T02-query.html>), SMART (<http://smart.embl-heidelberg.de/>), and superfamily (<http://supfam.mrc-lmb.cam.ac.uk/SUPERFAMILY/>) programs. Gene function was predicted based on annotation, expression pattern, and mutant phenotype available in FlyBase (<http://flybase.bio.indiana.edu>), WormBase (<http://www.wormbase.org>), and Ensembl (<http://www.ensembl.org>). The 36 genes known to be involved, or implicated, in ciliogenesis were identified by detailed searches of PubMed, OMIM, and the Protein databases.

Transgenic Constructs

The generation of the 17 OSEG promoter-Gal4 transgenes was performed by cloning upstream elements immediately adjacent to the predicted initiator methionine of individual *oseg* genes in front of a modified CasSper-AUG-Gal4 vector (Vosshall et al., 2000). The *oseg1* fragment was 6842 nucleotides; *oseg2*, 3115 nucleotides; *oseg3*, 1021 nucleotides; *oseg4*, 640 nucleotides; *oseg5*, 452 nucleotides; *oseg6*, 5002 nucleotides; BBS1, BBS2, BBS8, CG5142, CG7735, CG3259, CG14870, CG1126, CG5359, CG15161, CG9227, 1500 or 2000 nucleotides. Gal4 drivers, transformation rescue constructs, and GFP fusion proteins were cloned into the pP(UAST) vector. For translation fusions, GFP was fused at the C terminus of OSEG2, 3, 4, and 6 and at the N terminus of the OSEG1. We used an Elav-Gal4 driver (Lin and Goodman, 1994) as a pan-neuronal promoter in all our translational gene fusion experiments. In order to increase the signal from OSEG-GFP fusions inside the outer segments of chemo- or mechanosensory neurons, transgenic constructs were examined in animals carrying two copies of the Elav-Gal4 driver and the fusion construct.

Experimental Animals

Drosophila stocks containing ELAV-Gal4 (c1115), UAS-GFP, *Df(3L)66C-G28*, *Df(3L)66C-I65*, and *kfp64D^{kl}* were obtained from the Bloomington Stock Center; *Df(3L)Aprt-1* and *Df(3L)R-R2* from L. Goldstein; *EP(3)3161* from G. Rubin; UAS-GFP- α 1tub84B from A. Spradling (Grieder et al., 2000). Our control lines were isogenized *cn*, *bw*, and *bw; st* flies. P element-mediated germline transforma-

tions and fly manipulations were performed according to standard techniques. Rescue of *oseg1* and *oseg2* were assayed in ELAV-Gal4; UAS-OSEG1/Cyo; *oseg1* and ELAV-Gal4; UAS-OSEG2/Cyo; *oseg2⁴⁵²*, respectively.

Genetic Screen for Candidate Ciliary Mutants

Mutagenized lines were generated using isogenized *bw; st* flies; homozygous mutants are white eyed while heterozygous siblings are brown eyed. To identify pharate adult lethal lines, lightly populated vials were screened for the presence of white-eyed nonbalanced pupae, but also the absence of viable white-eyed adult flies. To test uncoordinated behavior, mutant pupae were transferred to humidified chambers and examined by visual inspection as described previously (Kernan et al., 1994). Electrophysiological recordings (Walker et al., 2000) and proboscis extension assays (Galindo and Smith, 2001) were performed within 8–24 hr after eclosion; for MRC recordings, the TEP was held at 40 mV. A minimum of four flies were examined for proboscis extension assays and a minimum of five bristles from three different flies were used for all electrophysiological recordings.

Imaging and Immunofluorescence Staining

Whole-mount preparations of pupal thorax or adult tissues were fixed in 4% formaldehyde in PBS, permeabilized by 0.3% Triton-X100, and blocked using 1% bovine serum albumin in PBS plus 0.3% Triton-X100 (blocking buffer). Samples were incubated in a 1:100 dilution of anti-GFP or 1:50 dilution of mab21A6 (Fujita et al., 1982) in blocking buffer for 12–18 hr at 4°C and detected using fluorescein-conjugated donkey anti-rabbit or Red-X-conjugated anti-mouse secondary antibodies (Jackson Immunolaboratory). Cuticular bristle organ structures (socket and shaft) were visualized by exciting the tissue with blue light and collecting autofluorescence signals in the red and green channels. Images were obtained using a BioRad MRC1024 confocal microscope with an argon-krypton laser. The identity of cells expressing GFP was established based on their morphology, location, innervation patterns, and, in the case of chordotonal organs, aided by the number of scolopoles.

Tissue Processing for Electron Microscopy

For EM analysis of mechanosensory macrochaetae, we studied the scutellar bristles; for chemosensory bristles, we examined the outer surface of the proboscis. Tissue was fixed either in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, with 5% sucrose, or in 4% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer with 2 mM calcium chloride and 1% tannic acid, pH 7.3, for 3–4 hr at 4°C. After washing in 0.1 M cacodylate or phosphate buffers, respectively, the specimens were postfixed in 1% OsO₄, *en bloc* stained with 1% uranyl acetate, dehydrated in ethanol series, and embedded in either Spurr or Polybed/Araldite (Polysciences). Ultrathin sections were post-stained with 2% aqueous uranyl acetate, triple lead stain, and examined with JEOL 1200 transmission electron microscope. A minimum of four bristles from three flies were examined for each mutant backgrounds.

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