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LETTERS

Transforming the architecture of compound eyes

Andrew C. Zelhof[1], Robert W. Hardy[1], Ann Becker[1] & Charles S. Zuker[1]

Eyes differ markedly in the animal kingdom, and are an extreme example of the evolution of multiple anatomical solutions to light detection and image formation. A salient feature of all photoreceptor cells is the presence of a specialized compartment (disc outer segments in vertebrates, and microvillar rhabdomeres in insects), whose primary role is to accommodate the millions of light receptor molecules required for efficient photon collection. In insects, compound eyes can have very different inner architectures[1–3]. Fruitflies and houseflies have an open rhabdom system, in which the seven rhabdomeres of each ommatidium are separated from each other and function as independent light guides. In contrast, bees and various mosquitoes and beetle species have a closed system, in which rhabdomeres within each ommatidium are fused to each other, thus sharing the same visual axis. To understand the transition between open and closed rhabdom systems, we isolated and characterized the role of *Drosophila* genes involved in rhabdomere assembly. Here we show that Spacemaker, a secreted protein expressed only in the eyes of insects with open rhabdom systems, acts together with Prominin and the cell adhesion molecule Chaoptin to choreograph the partitioning of rhabdomeres into an open system. Furthermore, the complete loss of *spacemaker* (*spam*) converts an open rhabdom system to a closed one, whereas its targeted expression to photoreceptors of a closed system markedly reorganizes the architecture of the compound eyes to resemble an open system. Our results provide a molecular atlas for the construction of microvillar assemblies and illustrate the critical effect of differences in a single structural protein in morphogenesis.

During ommatidium biogenesis the apical membranes of different photoreceptor cells separate from one another and concomitantly produce the rhadomeric structure needed for housing the phototransduction machinery. This process involves the precise coordination of membrane–membrane adhesion events both within and between rhabdomeres, and culminates in the production of about 60,000 microvilli per cell, each 1–2 μm in length and 50 nm in diameter[4,5]. The generation of the inter-rhabdomeral space (IRS; Fig. 1), by which rhabdomeres of the photoreceptor neurons partition from each other, is an essential event in the transition of compound eyes from a closed to an open system. This evolutionary change resulted in a considerable improvement in angular sensitivity, thus allowing the detection of smaller moving objects. We screened adult viable ethylmethane sulphonate-mutagenized *Drosophila* lines[6] for defects in rhabdomere structure and topology by examining deep-pseudopupil phenotypes[7,8] and performing an electron-microscopic ultrastructural analysis of pupal and adult eyes in candidate lines. Only 2 of 40 different complementation groups, *spacemaker* (*spam*) and *prominin* (*prom*), had eyes with apparently normal microvilli but showing a striking failure of the rhabdomeres to separate from each other, including a marked loss of IRS, irregular rhabdomere morphology and improper rhabdomeral contacts (Fig. 1a–d). Interestingly, loss-of-function mutations in *spam* completely eliminated the IRS, thus generating ommatidia resembling the compound eyes of animals with closed rhabdom systems (compare Fig. 1a–c).

To understand how *spam* and *prom* function, both mutants were mapped and the candidate genes were cloned and tested for rescue of the mutant phenotypes by P-element germline transformation (Supplementary Fig. 1c). *spam* encodes a 2,165-amino-acid polypeptide containing several protein motifs commonly found in extracellular molecules: an amino terminus consisting of seven epidermal growth factor (EGF)-like repeats, a linker region containing multiple sites for glycosaminoglycan addition, and a carboxy terminus including four alternating repeats of EGF-like and Laminin G domains (Supplementary Fig. 1a). *prom* encodes a 910-amino-acid Prominin (Prom)-like molecule (Supplementary Fig. 1b), a family of evolutionarily conserved transmembrane proteins often associated with microvilli[9] but of unknown function.

What is the role of *spam*? Sequence analysis of *spam* indicates that it might function as an extracellular protein (Supplementary Fig. 1). If Spacemaker (Spam) is a secreted molecule, it should localize to the IRS. Indeed, immunocytochemical studies showed that Spam is selectively localized to the IRS (Fig. 1g, h). However, mosaic analysis reveals that even though Spam is capable of diffusing throughout the ommatidium, near wild-type levels of the protein are still required to ensure complete rhabdomere separation (Supplementary Fig. 3b, and data not shown). To determine when *spam* function is required, we placed its transcriptional unit under the control of an inducible heat-shock promoter (UAS-*spacemaker* and heat shock (HS)-GAL4) and subjected mutant animals to temperature shifts at various times during development. Our results demonstrated that *spam* expression at 36–64 h after puparium formation (APF), a window of time coincident with the initiation of rhabdomere biogenesis, is sufficient and necessary for complete rescue of the phenotype (Supplementary Fig. 1c). Late expression (about 72 h APF), using either HS-GAL4 or a late-expressing rhodopsin promoter (Rh1-GAL4), leads only to a partial rescue: Spam is still secreted into the IRS and the apical stalk membranes (the base of the rhabdomeres) are still capable of separating, but the rhabdomeres themselves remain fused in the centre of the ommatidium (Supplementary Fig. 1c). Notably, this phenotype closely resembles that of *prom* mutants (Fig. 1d), suggesting that they might both participate in a common morphogenesis programme.

If *prom* and *spam* function in the same pathway, we would expect genetic interactions between these two loci. Indeed, although both *spam* and *prom* are recessive genes, the *spam prom* double heterozygote shows a failure of the rhabdomeres to separate and the loss of a continuous IRS (Fig. 1e); these results indicate that the two gene products probably act together in the biogenesis of the IRS (see below). To explore the function of Prom, we first examined its spatial and temporal expression pattern. Like Spam, Prom is present at the beginning of rhabdomere biogenesis (48 h APF), when it decorates the entire photoreceptor apical surface (Supplementary Fig. 2a). By the time of eclosion, however, Prom is selectively localized to the stalk membrane and the tips of the microvilli; this is best illustrated by simultaneous labelling with Chaoptin, a photoreceptor adhesion

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protein expressed throughout the perimeter of microvilli (Supplementary Fig. 2c, f, i). Given that Spam is an IRS-secreted molecule and Prom is a rhabdomere integral membrane protein, we examined whether these two proteins interact. When *Drosophila* tissue-culture cells are transfected with *spam*, the protein is secreted into the medium. However, if the same cells are instead transfected together with *prom*, Spam then localizes selectively to the exterior surface of the plasma membrane (Supplementary Fig. 4b). These results indicate that Prom might function as a binding partner for Spam. To demonstrate that Prom in fact recruits extracellular Spam to the plasma membrane, we performed a mixing experiment: RFP-labelled cells expressing *spam* were incubated with green fluorescent protein (GFP)-labelled cells transfected with *prom*. As proposed, secreted Spam now specifically localizes to the surface of Prom-expressing cells (Supplementary Fig. 4f). Taken together, these findings substantiate Prom as a candidate Spam receptor.

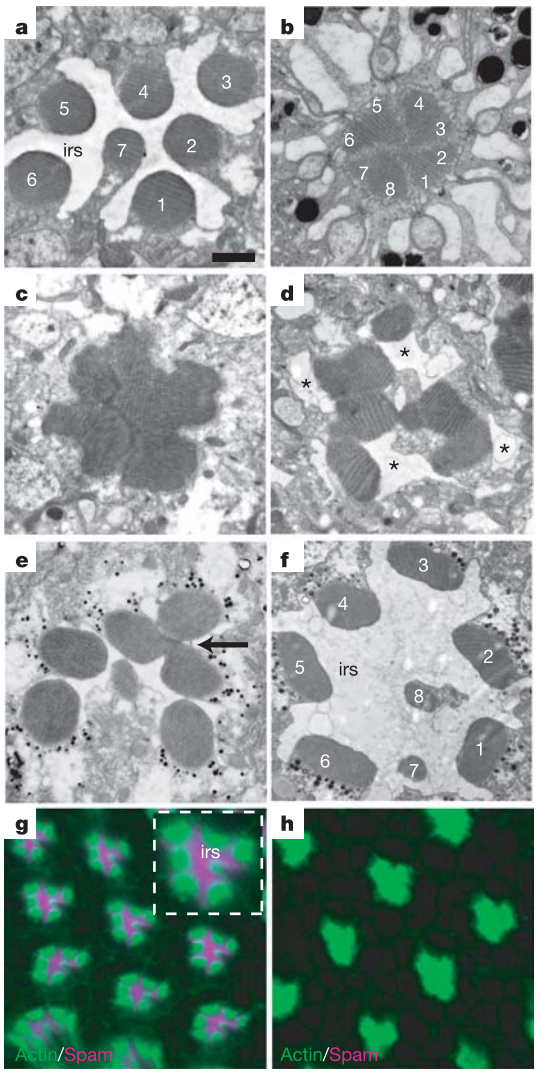

Figure 1 | *spam* and *prom* mutants have severe defects in IRS formation. **a–f**, Electron micrographs showing cross-sections through ommatidia of *Drosophila melanogaster* (**a**), *Apis mellifera* (**b**), *spam*^{1} (**c**), *prom*^{1} (**d**), *spam*^{1} +/+ *prom*^{1} (**e**) and flies overexpressing *spam* (GMR-GAL4, UAS-*spam*) (**f**). Asterisks and IRS mark the inter-rhabdomeral space and numbers label each rhabdomere. Note the absence of IRS in *spam* and *prom* mutants, and the incomplete rhabdomeric separation in the transheterozygote (arrow). Scale bar, 1 μm. **g**, Spam (magenta) expression in wild-type ommatidia; **h**, *spam*^{1}-null ommatidia. Note Spam localization to the IRS surrounding each of the rhabdomeres (microvilli are labelled green with phalloidin).

What is the function of *prom* and *spam* in orchestrating open rhabdomere development? Microvilli are interconnected by a network of homophilic interactions that ensures their tight and regular packing within rhabdomeres[10,11]. However, such an arrangement would also render microvilli vulnerable to inter-rhabdomeric adhesion. We suggest that secretion of Spam into the IRS forces the separation of the stalk membranes, pushing the rhabdomeres apart, and that the recruitment of Spam to the microvillar surface by the binding of Prom prevents inter-rhabdomere adhesion. This model is consistent with the phenotypes of *spam* and *prom* mutants, and makes three significant predictions: first, overexpression of *spam* should increase the volume of the IRS by pushing rhabdomeres further away from each other; second, in mosaic ommatidia containing *prominin* mutant cells there should be a significant loss of Spam binding and accumulation surrounding the mutant rhabdomeres; and third, the fused rhabdomeres of *prom* mutants should be rescued by independently reducing or abolishing inter-rhabdomere interactions. As predicted, overexpression of *spam* markedly expands the IRS of wild-type photoreceptors (Fig. 1f), and the presence of Prom in mosaic ommatidia promotes the recruitment of Spam selectively around wild-type rhabdomeres (Supplementary Fig. 3c, d). To alter inter-rhabdomere adhesion, we first needed to determine what molecule was responsible for the inappropriate fusion of rhabdomeres in *prom* and *spam* mutants. We reasoned that Chaoptin, a photoreceptor-specific glycosylphosphatidylinositol-linked membrane protein expressed very early during photoreceptor morphogenesis and required for crosslinking microvilli by means of homophilic interactions, might be a strong candidate[10–12]. We recognized that the early expression of Chaoptin would ensure the proper packing of microvilli within rhabdomeres and that the subsequent expression of Spam and Prom would guarantee that individual rhabdomeres remain separated in open rhabdom systems. Indeed, removing just one copy of the gene encoding Chaoptin (*chp*) in a *prom* mutant background (*prom*/*prom*;*chp*/+) is sufficient for a

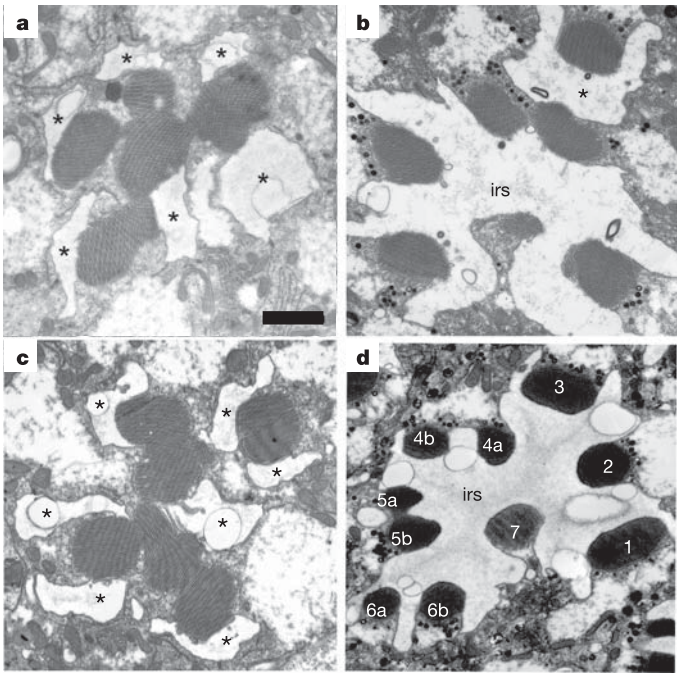

Figure 2 | Prom and Spam cooperate to antagonize the adhesive force of Chaoptin. **a–c**, Electron micrographs showing cross-sections through *prom*/*prom* (**a**), *prom*/*prom*;*chp*/+ (**b**) and *prom spam*/*prom* +;*chp*/+ (**c**) ommatidia. **d**, Early expression of Spam produces severely split rhabdomeres. Asterisks denote the IRS. Scale bar, 1 μm. See Supplementary Fig. 5 for a quantitative analysis of the data.

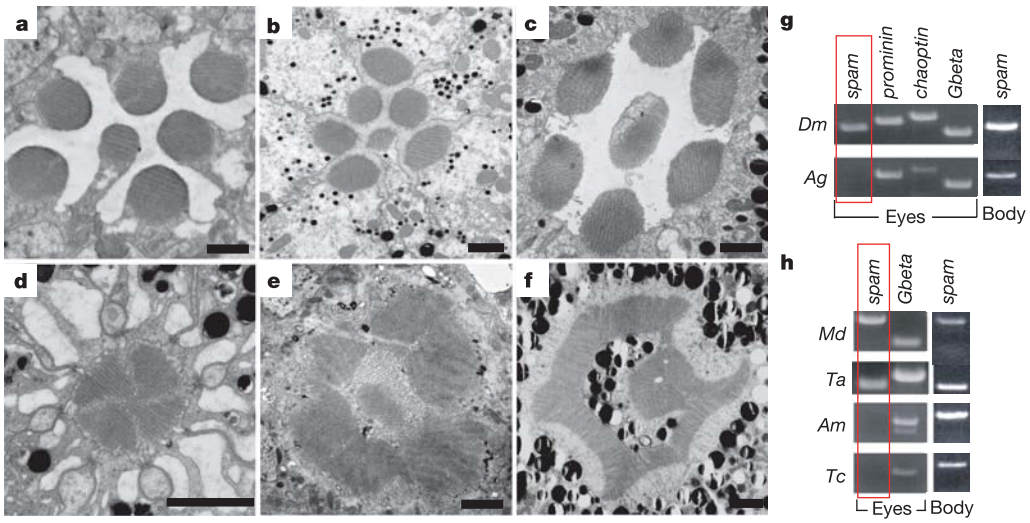

Figure 3 | *spam* is expressed only in insect eyes with an open rhabdomere system. **a**–**f**, Electron micrographs showing ommatidia of *Drosophila melanogaster* (*Dm*; **a**), *Musca domestica* (*Md*; **b**), *Toxorhynchites amboinenesis* (*Ta*; **c**), *Apis mellifera* (*Am*; **d**), *Tribolium castaneum* (*Tc*; **e**) and *Anopheles gambiae* (*Ag*; **f**). Scale bars, 1 μm. **g**, **h**, RT–PCR reactions examining the expression of *spacemaker*, *prominin*, *chaoptin* and *eye Gbeta* control mRNA in the pupal visual system and bodies of the various species. Multiple Spam-specific primers were designed for each species after the cloning of the corresponding homologues.

drastic suppression of the *prom* mutant phenotype (Fig. 2a, b and Supplementary Fig. 5). These data demonstrate that the capacity of Spam to push rhabdomeres apart is very sensitive to the amount of adhesive force available (for example Chaoptin). Given these results, we anticipated that further reducing the levels of *spam* in this *prom*/*prom*;*chp*/+ genetic background (that is, bringing the ratio of *spam* to *chp* back to normal levels) should revert the 'rescued phenotype' to the mutant state. As proposed, the rhabdomeres are now stuck together (Fig. 2c and Supplementary Fig. 5). Thus, in this genetic background, there is sufficient Spam to partition the stalk membranes but not enough to overcome the Chaoptin-dependent adhesiveness between adjacent rhabdomeres. A final prediction of these findings is that early expression of Prom and Spam should result in the development of broken-up rhabdomeres, probably as the Spam/Prom complex outcompetes the adhesive force linking microvilli. The data shown in Fig. 2d soundly validate this postulate. Together, these results demonstrate that Spam, Prom and Chaoptin orchestrate the assembly of microvilli, ensure the structural integrity and the partitioning of rhabdomeres, and guarantee the construction of an open rhabdom system.

Are these molecules responsible for the transformation between closed and open rhabdom systems? To examine this possibility we investigated the expression of Spam, Prom and Chaoptin homologues in other insect species containing open or closed rhabdoms (Fig. 3a–f). First, analysis of *Anopheles gambiae* eyes, a closed system, showed that both Prom and Chaoptin are present but Spam is absent from pupal eyes, suggesting that Spam might be the critical component for partitioning rhabdomeres (Fig. 3g). To lend further credibility to our hypothesis, we isolated and examined *spam* expression in several other species, two with open rhabdomere systems (the housefly *Musca domestica* and the mosquito *Toxorhynchites amboinensis*) and two with closed ones (the honeybee *Apis mellifera* and the flour beetle *Tribolium castaneum*). As predicted, Spam is conspicuously absent from the eyes of insects with closed rhabdomere systems (Fig. 3h), even though *spam* expression is still detected in the body of all of these species, probably reflecting an evolutionarily conserved role[13] (Fig. 3g). Taken together, these results substantiate *spam* expression as a powerful indicator of visual system architecture and suggest that targeting *spam*/*prom* function to a closed rhabdom system may well transform it into an open system. To test this final postulate, we focused on the ocellar photoreceptors of *Drosophila*. Ocelli are simple eyes located at the vertex of the fly head and involved in navigation[14,15]. They are composed of 70–90 photoreceptor neurons organized in a near-close rhabdomeric arrangement (Fig. 4a, d; note the lack of an IRS). We engineered flies in which

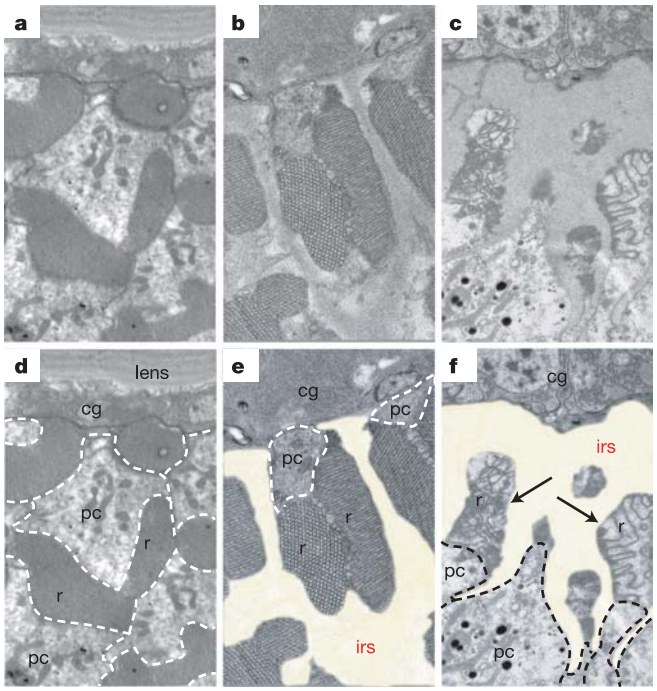

Figure 4 | Expression of Spam induces the formation of an IRS. Electron micrographs showing cross-sections through wild-type ocelli (**a**, **d**), ocelli overexpressing Spam, *spam*/*spam*;HS-GAL4/UAS-*spam* (**b**, **e**) and ocelli strongly overexpressing Spam, GMR-GAL4/UAS-*spam* (**c**, **f**). Note the drastic reorganization of the ocellar visual system with increasing amount of Spam (see text for details). The bottom panels highlight the IRS (coloured in yellow). Arrows denote the presence of disassembled microvilli within individual rhabdomeres, and the dotted lines outline the body of the photoreceptor cells. Abbreviations: pc, photoreceptor cell; r, rhabdomere; cg, corneagenous cells.

Spam expression was targeted to the ocelli under the control of either a weak (HS-GAL4/UAS-*spam* at 29 °C) or a strong (glass multimer reporter (GMR)-GAL4, UAS-*spam*) promoter, and examined eye morphology by electron microscopy. Overexpression of Spam produced a complete reorganization of ocellar eye architecture ranging from the creation of a novel IRS surrounding otherwise structurally intact rhabdomeres (Fig. 4b, e) to the generation of an extensive IRS accompanied by the disintegration of the rhabdomeres into their individual microvilli (GMR–GAL4, UAS-*spam*) (Fig. 4c, f).

Taken together, these studies delineate a vital evolutionary role for Spam in the construction of insect eyes, assign candidate functions to three important proteins and provide a mechanistic path for the assembly of rhabdomeres. Interestingly, human prominin-1 localizes to the base of the outer segments, a subcellular compartment responsible for producing new discs, and frameshift mutations in hProm-1 leads to severe retinal degeneration[16]. We suggest that, much as in *Drosophila*, Prom has a key function in the morphogenesis of discs by ensuring their unimpeded folding and stacking into the outer segment.

METHODS

Genetic stocks/experimental animals. The following stocks were used in this study: *cn bw*, Heat Shock-GAL4, RH1-GAL4, GMR-GAL4, *chp*[2], *prom*[1], and *spam*[1]. FRT Stocks: ey-FLP;GMR-myrGFP FRT40A, ey-FLP;GMR-myrGFP FRT42D, *spam* FRT40A and *prom* FRT42D. P-element-mediated germline transformation and fly manipulations were performed in accordance with standard techniques.

Electron microscopy, immunofluorescence staining, and imaging. Insect heads were fixed in 4% formaldehyde, 3.5% glutaraldehyde, 100 mM cacodylate buffer (pH 7.4), 2 mM CaCl for 3 h at 22 °C, and then fixed again overnight at 4 °C. Heads were rinsed in 100 mM cacodylate buffer and postfixed in 2% buffered osmium for 1 h at 22 °C. The heads were dehydrated through an ethanol series, rinsed with propylene oxide and embedded in EMBED-812 for sectioning. After counterstaining, the sections were viewed and photographed with a Jeol 1200EX II transmission electron microscope. Developing whole retinas were dissected at the appropriate time and processed as described previously[17]. Creation and processing of frozen thin sections were performed as described[18]. The antibodies used were as folows: Prom, rabbit polyclonal antibody produced by injecting rabbits with the C-terminal peptide ARSKGRHRRSDRSP; Spam, in this study we have shown that the previously unknown antigen for mAb21A6 (ref. 19) is Spam; Chaoptin, mAb2A12 (ref. 19). Rhodamine–fluorescein isothiocyanate (FITC)-conjugated phalloidin (Invitrogen) was used for the detection of F-actin. FITC-conjugated, Cy5-conjugated and Red-X-conjugated secondary antibodies were obtained from Jackson ImmunoResearch Laboratories. All images were captured on a Bio-Rad MRC1024 or Zeiss LSM510 confocal microscope and imported into Adobe Photoshop. Antibodies were tested and validated for specificity and selectivity by performing staining reactions in control and loss-of-function mutant alleles. The anti-Spam, anti-Prominin and anti-Chaoptin[11] staining is completely abolished in null alleles (see Fig. 1h, Supplementary Table 1 and Supplementary Fig. 3a).

Transgenic constructs. Complementary DNAs representing *spacemaker* and *prominin* were cloned into pUAST[20] and transformed into *Drosophila*. The rescue of *spacemaker* and *prominin* was assayed in *spam*/*spam*;HS-GAL4/UAS-*spam*, *spam*/*spam*;RH1-GAL4/UAS-*spam*, *prom*/*prom*;HS-GAL4/UAS-*prom*, and *prom*/*prom*;RH1-GAL4/UAS-*prom* and reared at 22 °C. For the temperature-shift experiments white puparia were collected and transferred to 29 °C at various times of development and assayed by electron microscopy for rescue. For overexpression of Spacemaker HS-GAL4/UAS-*spam* larvae were collected, heat shocked for 1 h at 37 °C every 24 h and reared at 29 °C until eclosion.

Transfection assays. *Drosophila* Schneider 2 cells were transfected with Effectene (Qiagen), and 48 h after transfection they were plated on polylysine-coated slides for fixing and imaging. Cells were transfected with pUAST–Spacemaker, pUAST–Prominin, pUAST–GFP and pUAST–mRFP and to drive expression they were co-transfected with pTub–GAL4 (ref. 21). All fixations and staining were performed in the absence of detergent to detect the surface localization of Spam.

RNA isolation and RT–PCR. Extraction of total RNA from insect tissues was performed with Trizol (Invitrogen). In addition, all RNA samples were treated with DNase and first-strand synthesis was primed with oligo(dT) and completed with Superscript First Strand Synthesis (Invitrogen). Species-specific primer sets were used for PCR amplification (see Supplementary Table 2).

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Information The cDNA sequences for *spacemaker* and *prominin* are deposited in GenBank under accession numbers DQ780942 and DQ780943, respectively. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Correspondence and requests for materials should be addressed to C.S.Z. (charles@flyeye.ucsd.edu) or A.C.Z. (azelhof@flyeye.ucsd.edu).

Transforming the architecture of compound eyes

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Supplementary Material

Supplementary Figure 1: Deduced genomic and protein organization of *spam* and *prom*. **a,** *spacemaker* locus. The *spam* transcript consists of 13 exons spread over 34kb of DNA. The gene is located at chromosome position 22E1 and includes portions of previously annotated genes CG15388, CG15389, and CG7245. The localization and nature of the *spam* mutations (*spam*[1-6]) are shown on the genomic and protein map. The size of the "hash" marked introns are indicated below each intron. Agrin, an extracellular matrix protein involved in the formation of the neuromuscular junction[1], and Perlecan, a component of most basement membranes, display prominent domain similarity to the C-terminus of Spacemaker[2]. **b**, *prominin* locus. The *prom* transcript consists of 11 exons. *prom* is allelic to *eyes closed* (*eyc*)[3] and located at chromosome position 60C8. However, CG4556, the mis-identified transcript for *eyc* is located entirely within the first intron of the *prom* transcript. The *prom* transcript includes portions of previously annotated genes CG30166 and CG30164. The localization and nature of the mutations of *prom* and *eyc* are indicated on the genomic map. RT-PCR was used to confirm transcript size and organization for both loci. cDNAs encoding the above transcripts rescue the corresponding mutant phenotypes, **c**. Scale bar 1 um. Mutants were mapped first using RFLP, and PLPs strategies[4]**,** and then fined mapped using small deficiencies and P-element insertions. The EMS-induced molecular lesions were identified by sequencing genomic DNA isolated from homozygous mutant alleles and control *cn bw* chromosomes.

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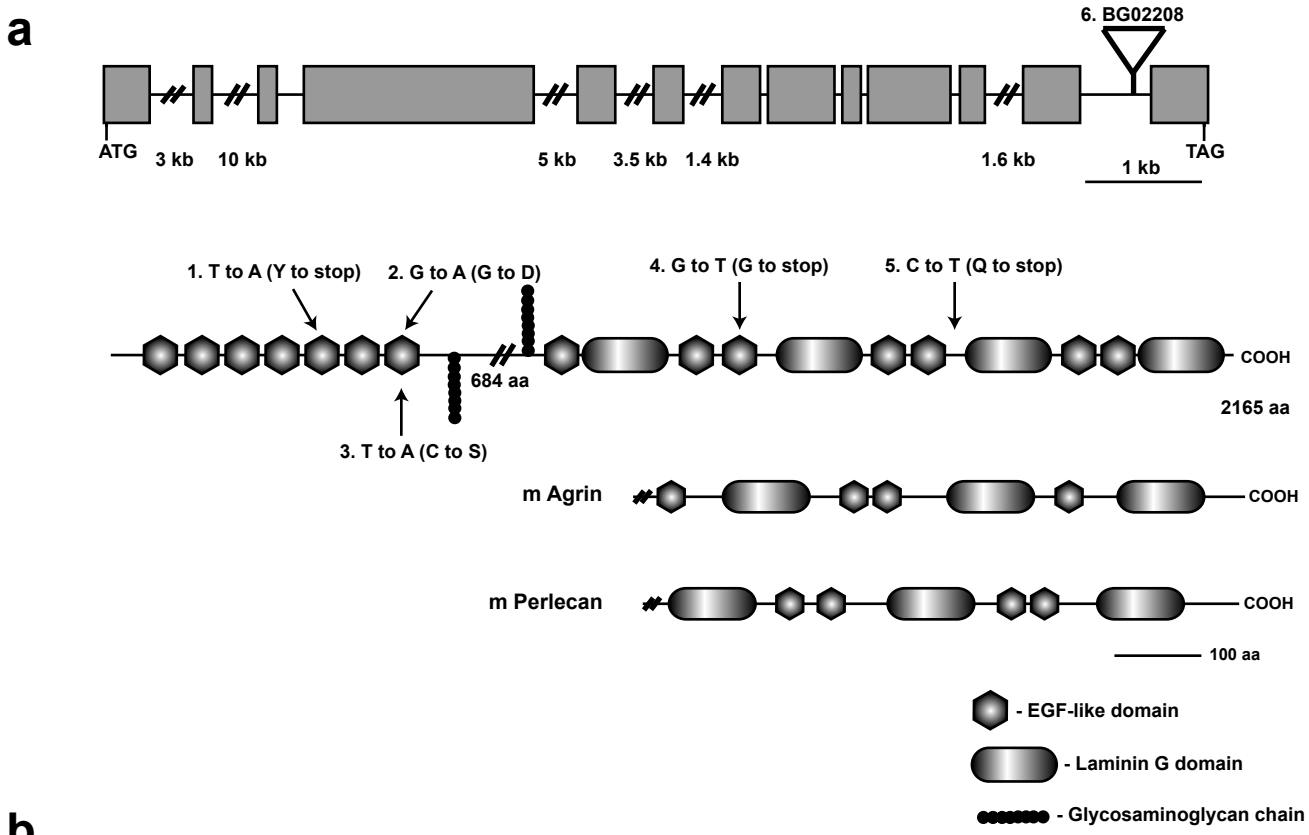
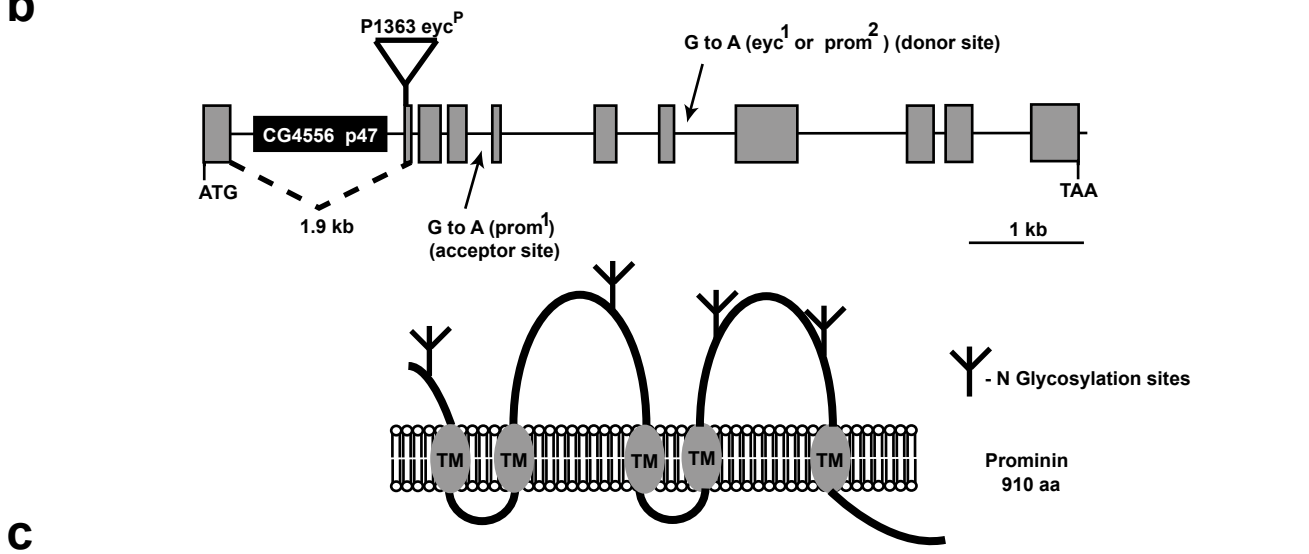
Supplementary Figure 1





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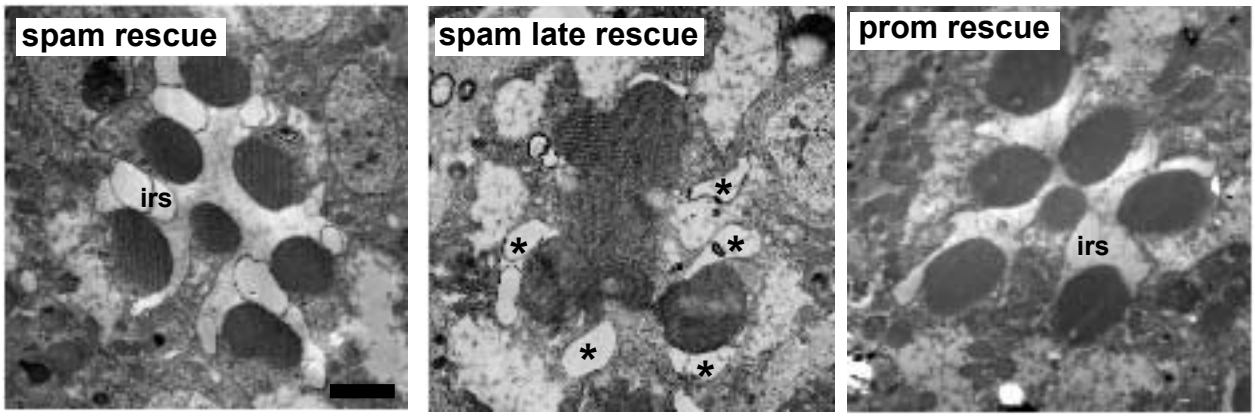
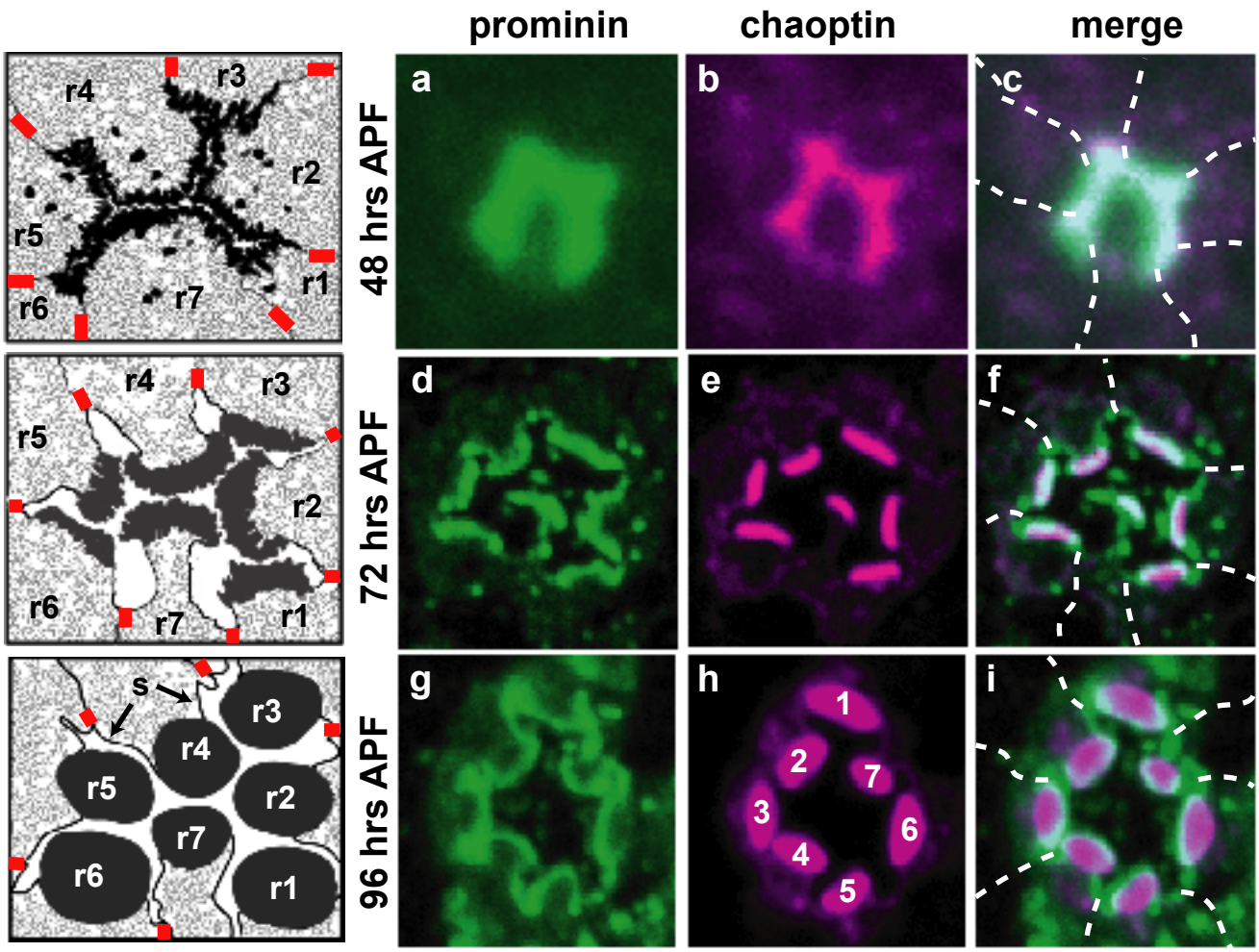
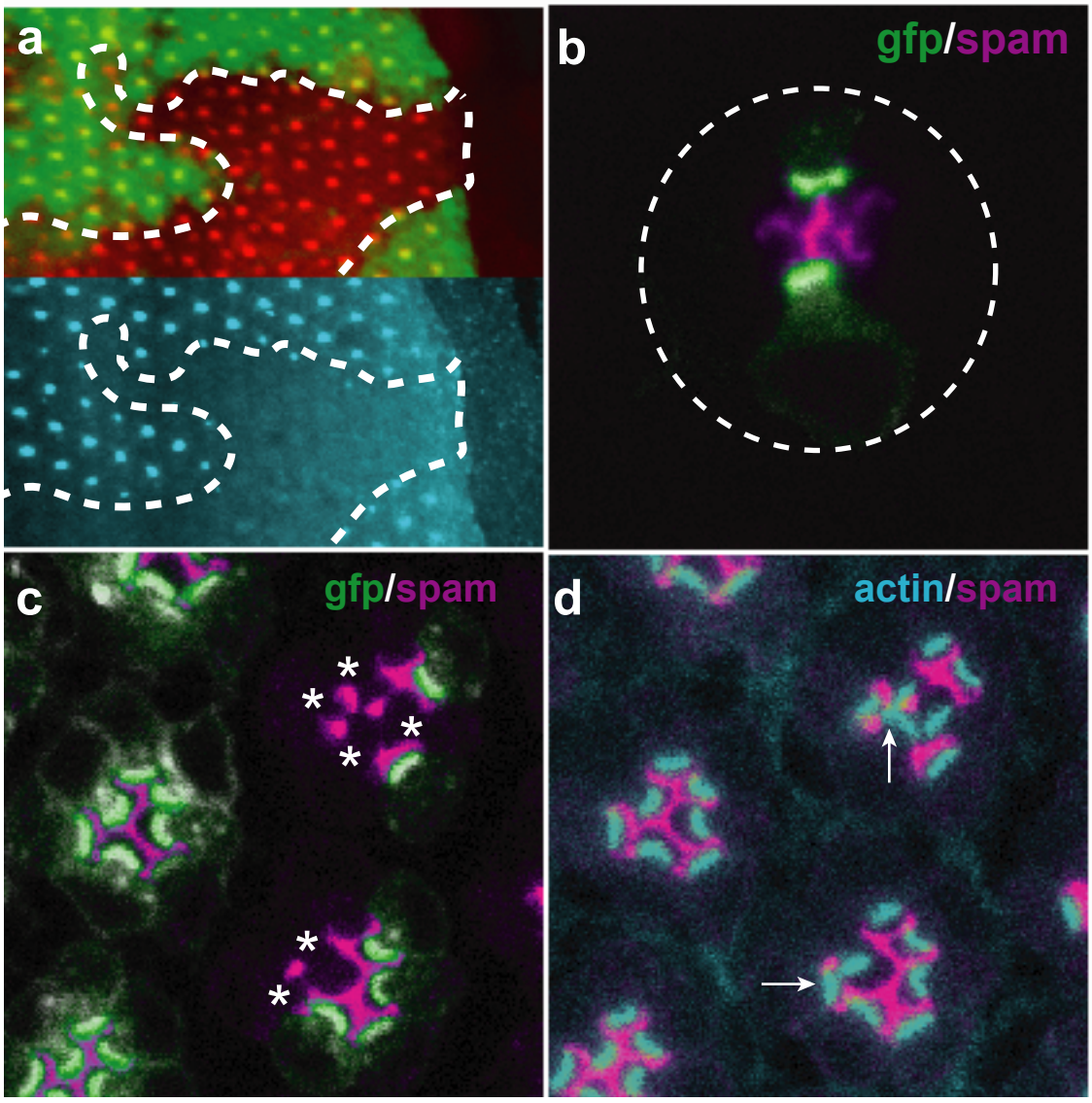
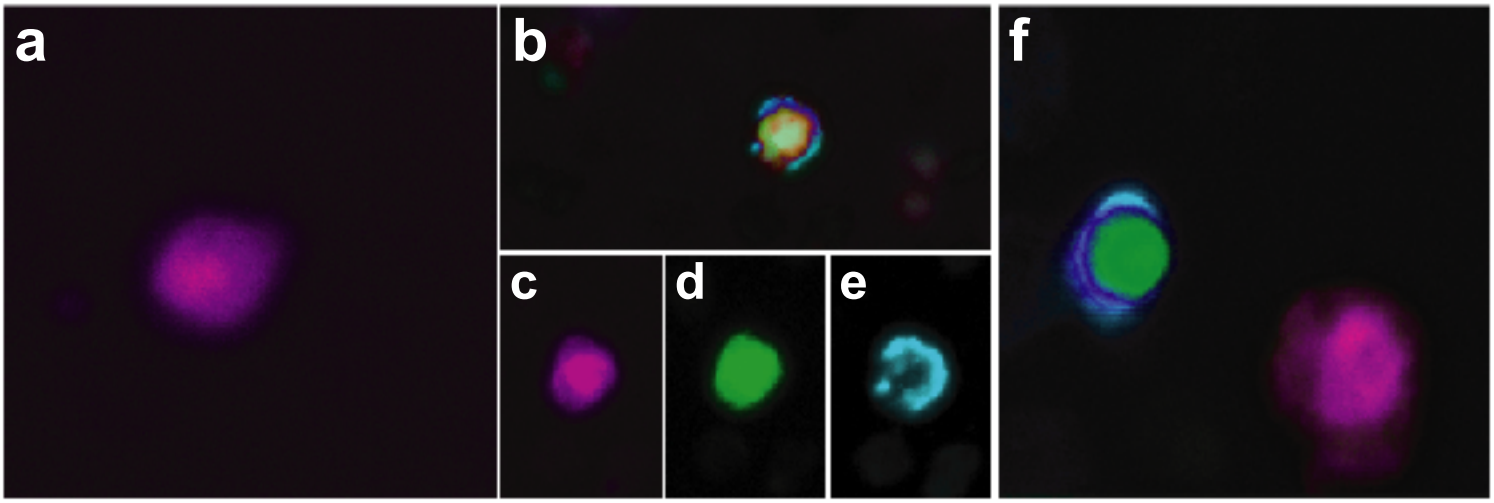




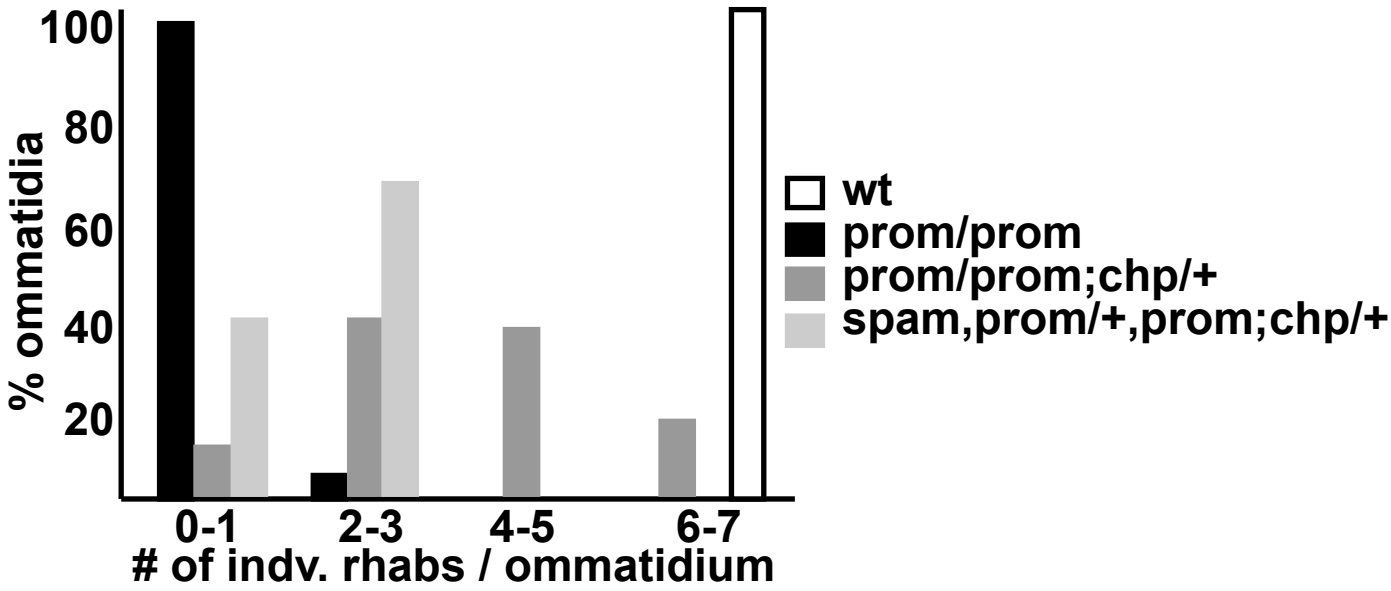
Supplementary Figure 2 Prominin and Chaoptin localize to distinct domains during rhabdomere development. a-c, At 48 hrs APF, Prominin (green) and Chaoptin (magenta) show complete colocalization on the apical surface of photoreceptor cells. **d-f**, By 72 hrs APF, these two proteins have started to segregate into distinct regions of the apical membrane. **g-i,** Prior to eclosion, Prominin localization is limited to the apical regions of microvilli and the apical stalk membranes, while Chaoptin is expressed throughout the microvillar surface. Dotted lines delineate the boundaries between photoreceptor cells. Left panels show diagrams of rhabdomere morphogenesis at the equivalent times [5]; red boxes represent adherens junctions between photoreceptor cells, s-stalk membrane.



Supplementary Figure 3 Mosaic analysis of *spam* and *prom* function. **a**, Mosaic compound eye, with wildtype *prominin* photoreceptor cells marked in green (GFP), rhabdomeres in red (phalloidin), and Prom antibody labeling in blue. Note the total lack of Prom staining in mutant cells (*prom*^{1} allele). The dotted line outlines the clonal boundary. See Fig. 1h for a corresponding test of the specificity of anti-Spam antibodies used in this study. **b**, Mosaic ommatidium, with wildtype Spam-producing photoreceptor cells marked in green. Note diminished Spam (magenta) allocation throughout the IRS. The dotted line outlines a single ommatidium. **c**, Spam distribution in *prom* mosaic ommatidia. The presence of Prom ensures a rhabdomere is surrounded by a crescent of Spam (magenta) accumulation; wildtype rhabdomeres are labeled with GFP. **d**, Panel in b was labeled with phalloidin to highlight the location of rhabdomeres (blue). Asterisks mark mutant rhabdomeres in which Spam accumulation is either missing or abnormal, and arrows show fused rhabdomeres due to the lack of Prominin.



Supplementary Figure 4 Prominin recruits Spacemaker to the plasma membrane. **a,** Drosophila S2 cell co-transfected with RFP and *spam* and stained for surface Spam expression. No Spam is detected on the surface of the cell. **b-f,** Spam is recruited to the surface of Prom-expressing cells. S2 cells were co-transfected with Prom+GFP and Spam+RFP reporters. Spam selectively decorates the surface of Prom expressing cells: **b**, composite figure, **c**, RFP staining, **d**, GFP staining, **e**, Spam antibody staining (blue). **f**, When S2 cells expressing RFP (red) and *spam* are mixed with cells expressing GFP (green) and *prom* , secreted Spam (blue) selectively accumulates on the surface of the Prominin expressing cells.



Supplementary Figure 5 Prominin and Spacemaker antagonize the adhesive force of Chaoptin. Electron micrographs of cross sections through *prom*/*prom*, *prom*/*prom*; *chp*/+, and *prom*, *spam*/*prom*, +; *chp*/+ ommatidia were analyzed for the presence of individually separated rhabdomeres. As expected, wild type flies (open bars) have all of their rhabdomeres clearly segregated, while most (if not all) rhabdomeres fail to separate in *prom* homozygous mutants (back bars). However, a reduction in the levels of Spam or Chp in a *prom* mutant background dramatically alters the phenotype. See text and figure 2 for details. A minimum of fifty ommatidia from at least two different heads were examined for each genotype.

Supplementary Table 1 Sequence characterization of Spam and Prom alleles. The table details the molecular lesion in the various alleles. Position 1 is defined as the first nucleotide of the ATG initiator methionine for each protein. *spam*^{1}, *prom*^{1} and *prom*^{2} are protein null alleles. *spam*^{2} and *spam*^{3} show nearly normal levels of Spam but the protein fails to be secreted. Expectedly, these flies display a severe loss of IRS. *spam*^{4}, *spam*^{5} and *spam*^{6} are hypomorphic alleles, with a corresponding reduced level of antibody labeling and less severe loss of IRS.

Allele	**DNA Change**	**Amino Acid Change**
spam^{1}	T^{957} to A	Y^{319} to stop codon
spam^{2}	G^{1136} to A	G^{379} to D
spam^{3}	T^{1174} to A	C^{392} to S
spam^{4}	G^{3931} to T	G^{1311} to stop codon
spam^{5}	C^{5041} to T	Q^{1683} to stop codon
spam^{6}	p-element insertion BG02208	C-terminal truncation
prom^{1}	**G**/A^{764} to A	acceptor site/ null
prom^{2}	T^{1226}/**G** to A	donor site/null

Supplementary Table 2 Species specific primer sets used for PCR amplification.

Species	Gene	5' Primer	3' Primer
Dm	*spam*	ACCTTTACTTGCATCTGCCAGGAG	CGGAACTCCACCACTCCGCCCTGCAGC
Ag	*spam*	ACGTTCACGTGCATCTGCCAGGAG	CGGTACTCCACCACGCCTCCCTGCAGG
Md	*spam*	TACGGTCCATTGTGTGCCCAGCCC	CAGCGATACGAAGCCGATCTTCTT
Tc	*spam*	GGTTACATCATGCTGACGTGGAATCTG	CTTTCCAATCGGACAATCGCACTCATA
Am	*spam*	GGTACTTGTACCTGGATTCTCATC	CCACGACCTGTTGCAGGGCTTGAA
Ta	*spam*	ATCTACCGTTGCATTCGGGCTTCCAA	AGATATTTGTGGGAAACAGCTAGCAT
Dm	*prom*	CGCCCGCTCTGCGACACGTTGCGTAT	ACGCAGTAGCTCTCCGATGATGCC
Ag	*prom*	CGGCCGCTTTGTGATACGCTGCGCAT	TCGCAGCAGCTCCCCGATGATCCC
Dm	*chp*	GAGCCCTACTTCCTGCAGTCCACGGGC	GGTGAGATTGTTGCCAGACAAATC
Dm	*chp*	GAACCATACTTCCTACAGTCCACCGGC	GGTCAGATTGTTGCCGGACAGATC
Dm	*Gbeta*	GGCAAGCTGATCATCTGGGACACCTGG	GGCGAACCCGAAGCCACTGGGATG
Ag	*Gbeta*	GGCAAGCTGATCATCTGGGACACCTGG	AGCAAACCCGAATCCACTCGGATG