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SHORT REPORT

Does Unc–GFP uncover ciliary structures in the rhabdomeric eye of *Drosophila*?

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ABSTRACT

The *uncoordinated* (*unc*) gene product, a potential ortholog of mammalian orofaciodigital syndrome 1 (Ofd1), is involved in the assembly of the ciliary axoneme in *Drosophila* and it is, therefore, constrained to cell types that have ciliary structures, namely type 1 sensory neurons and male germ cells. Here, we show that evenly spaced Unc–GFP spots are present in the eye imaginal discs of third-instar larvae. These spots are restricted to the R8 photoreceptor cell of each ommatidium in association with mother centrioles. This finding is unexpected because the *Drosophila* eye is of the rhabdomeric type and would be expected to lack ciliary structures.

KEY WORDS: Rhabdomeric eye, Ciliary structures, Unc expression, *Drosophila*

INTRODUCTION

The *Drosophila* eye consists of ommatidial units derived from a simple epithelium of undifferentiated proliferating cells, the eye-antennal disc (Gehring, 1966; Haynie and Bryant, 1986). The patterning of the ommatidia begins in early third-instar larvae (Kumar, 2012) when the eye disc is crossed by a deep indentation, the morphogenetic furrow, that is formed because of the apical constriction of the epithelial cells (Ready et al., 1976). The progression of the morphogenetic furrow from the posterior to the anterior region of the disc delineates the differentiation of the ommatidial units (Wolff and Ready, 1991).

Eakin (1965) recognized two main morphological eye types, the ciliary form, exemplified by the eye of chordates, which is composed by photoreceptor cells displaying a 9+0 cilium, and the rhabdomeric form, typical of the invetebrate eye, lacking ciliary structures. However, these models are not so rigid and intermediate conditions sporadically occurred during the development of the rhabdomeric photoreceptors of some protostomes (Vanfleteren, 1982). Rudimentary cilia have, indeed, been occasionally observed in the rhabdomeric eyes of some onychophoran (Mayer, 2006), arachnid (Muñoz-Cuevas, 1975) and insect (Home, 1972, 1976) species.

We therefore asked whether the *Drosophila* ommatidia contained ciliary structures by looking at the distribution of an Uncoordinated (Unc) GFP fusion protein, a good marker for centriole-to-basal body conversion in *Drosophila* (Baker et al., 2004; Enjolras et al., 2012). The Unc protein has, until now, been found in the cell types in which the centrioles nucleate a ciliary axoneme, namely sensory neurons and male germ cells, but it is absent in other *Drosophila* tissues that lack ciliary structures. Surprisingly, we found distinct Unc–GFP signals within the developing photoreceptor clusters of the third-instar larval eye imaginal discs. Given that *Sas4* mutants that develop without centrioles have normal eye imaginal discs, it is conceivable that the Unc-associated centriole does not reflect functional activities. Rather, this centriole might represent the remnant of a vestigial structure present in the fly ancestor.

RESULTS AND DISCUSSION

An Unc–GFP signal was not found in the undifferentiated cells anterior to the morphogenetic furrow and through the mitotic waves (Fig. 1A). However, posterior to the morphogenetic furrow a striking localization of Unc–GFP in regularly spaced spots was observed (Fig. 1A). Remarkably, we found only one spot for each ommatidial cluster. This spot was always associated with the apical region of the posterior photoreceptor cell that contained more acetylated tubulin (Fig. 1B). Unc–GFP spots were also observed in the narrow region between the morphogenetic furrow and the second mitotic wave, where small groups of cells were arranged in large arcs or formed ommatidial pre-clusters (Fig. 1C).

Interestingly, the localization of Unc–GFP is consistent with the behaviour of R8, the first differentiating rhabdomeric cell that plays a pivotal role in the ommatidia assembly (Frankfort and Mardon, 2002; Pepple et al., 2008). The Unc–GFP signal indeed first appears in the more posterior cell of the arc-like clusters at the beginning of ommatidia formation. The Unc–GFP spot then moved toward the center of the ommatidia as they reached their complete photoreceptor cell number.

To verify whether the Unc–GFP expression was localized within the R8 cells, we counterstained the eye imaginal discs with an antibody against the Bridge of Sevenless (Boss) ligand that is specifically expressed on the apical surface of the R8 photoreceptors (Krämer et al., 1991). We find, indeed, that Unc–GFP colocalized with the Boss signal (Fig. 1D,E). However, Unc appeared just posterior to the morphogenetic furrow before the Boss ligand was expressed in R8 cells (Fig. 1D,E). This agrees with findings that Boss expression begins in row 3 (Krämer et al., 1991).

To confirm that the Unc–GFP spots observed in the ommatidial clusters could correspond to true centrioles, we co-immunolabeled the eye discs with an antibody against the centriole marker pericentrin-like protein (PLP) (Martinez-Campos et al., 2004). This antibody readily recognized distinct centriole pairs in the apical region of each photoreceptor cell (Fig. 2A). One PLP spot within each ommatidium overlapped the Unc–GFP signal (Fig. 2B), pointing to the association of the Unc protein with true centrioles. However, the Unc signal did not exactly overlap the PLP staining (Fig. 2C) suggesting that these proteins were expressed in two close regions. Similar observations have been reported during male spermatogenesis where PLP is found at base of the centriole, and Unc–GFP is localized to the mid-distal region of the centriole and to the cilium-like projections (Riparbelli et al., 2012). The Unc–GFP

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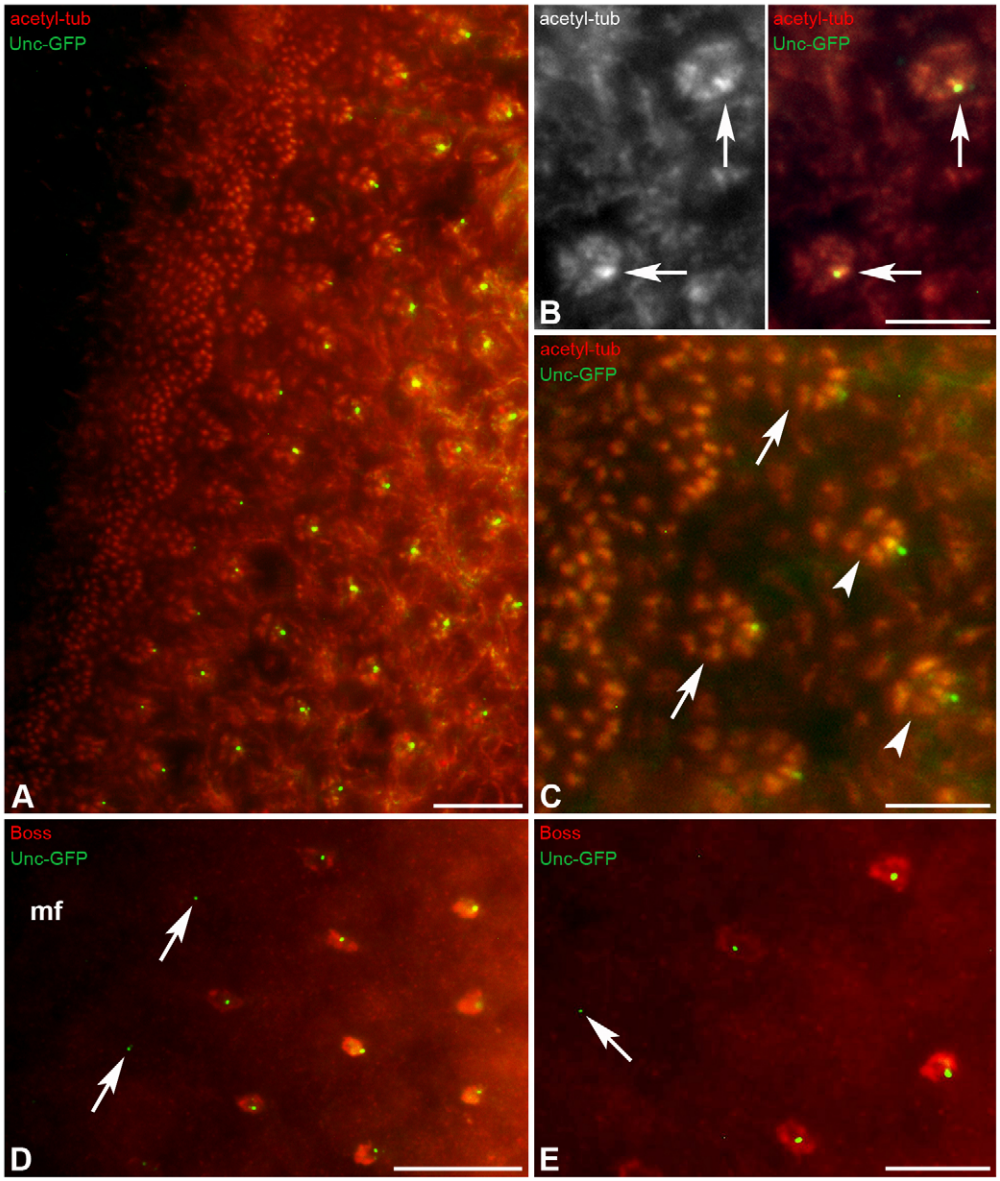

Fig. 1. Unc expression in the R8 photoreceptor cells. (A) Unc–GFP spots are closely associated with the ommatidial clusters but are lacking in the anterior region of the disc. (B) The Unc–GFP spots associate in each ommatidium with the more posterior photoreceptor cell showing higher acetylated-tubulin concentration (arrows). (C) Unc is not found in the morphogenetic furrow and is expressed in early differentiating cells at the arc stage (arrows) and in ommatidial preclusters (arrowheads). (D,E) Unc–GFP (green) colocalizes with the Boss expression (red) starting from the third ommatidial row, but only Unc dots (arrows) are visible just posterior the morphogenetic furrow (mf). Anterior is to the left. Scale bars: 10 µm (A,D), 5 µm (B,C,E).

dot was always associated with the stronger PLP spot (Fig. 2D). Given that PLP is more concentrated around the mother centriole in somatic *Drosophila* tissues (Fu and Glover, 2012; Richens et al., 2015), we asked whether the Unc signal was restricted to the mother centriole in the developing eye. Thus, we look at the distribution of centrobin, a good marker for daughter centrioles in *Drosophila* (Januschke et al., 2013). Upon co-immunolabeling the eye imaginal discs with antibodies against PLP and centrobin (Fig. 2E), we observed that only one centriole of each pair was stained for centrobin (Fig. 2F). This confirms that the anti-centrobin antibody is also a good marker to visualize daughter centrioles in the developing *Drosophila* eye. The Unc–GFP signal did not overlap the anti-centrobin staining (Fig. 2G,H), suggesting that the centrioles expressing Unc–GFP are mother centrioles.

Given that the Unc–GFP would be associated with centrioles that nucleate a ciliary axoneme (Baker et al., 2004) we performed a serial section analysis. Ommatidia were easily recognizable within the epithelium because their photoreceptor cells were arranged in racket-like clusters with the nuclei in the apical cytoplasm. To exclude the possibility that ciliary structures might escape our observation because they might be present in a very restricted developmental period, we analyzed the imaginal discs just at the posterior margin of the morphogenetic furrow where the Unc–GFP firstly appeared, and in the region close to the second mitotic wave, where the Unc–GFP signal was more evident. We examined serial sections of 63 whole ommatidial clusters from 17 different eye larval discs and we never observed ciliary structures but only encountered single or paired centrioles at the surface of the photoreceptor cells. However, negative results are difficult to verify and we cannot exclude that rudimentary ciliary structures might have escaped our analysis. Rather, we found that the posterior photoreceptor cell within each ommatidial cluster had a pair of centrioles arranged in tandem (Fig. 2I–L). This arrangement is similar to that of the centrioles found at the base of the ciliary axoneme in sensory neurons.

Given that a portion of the Unc–GFP signal is associated with the transition zone of the axoneme in *Drosophila* ciliated cells, we asked whether such a structure was present in the developing ommatidia. We therefore assessed the localization of Chibby, a protein selectively expressed in the transition zone of the

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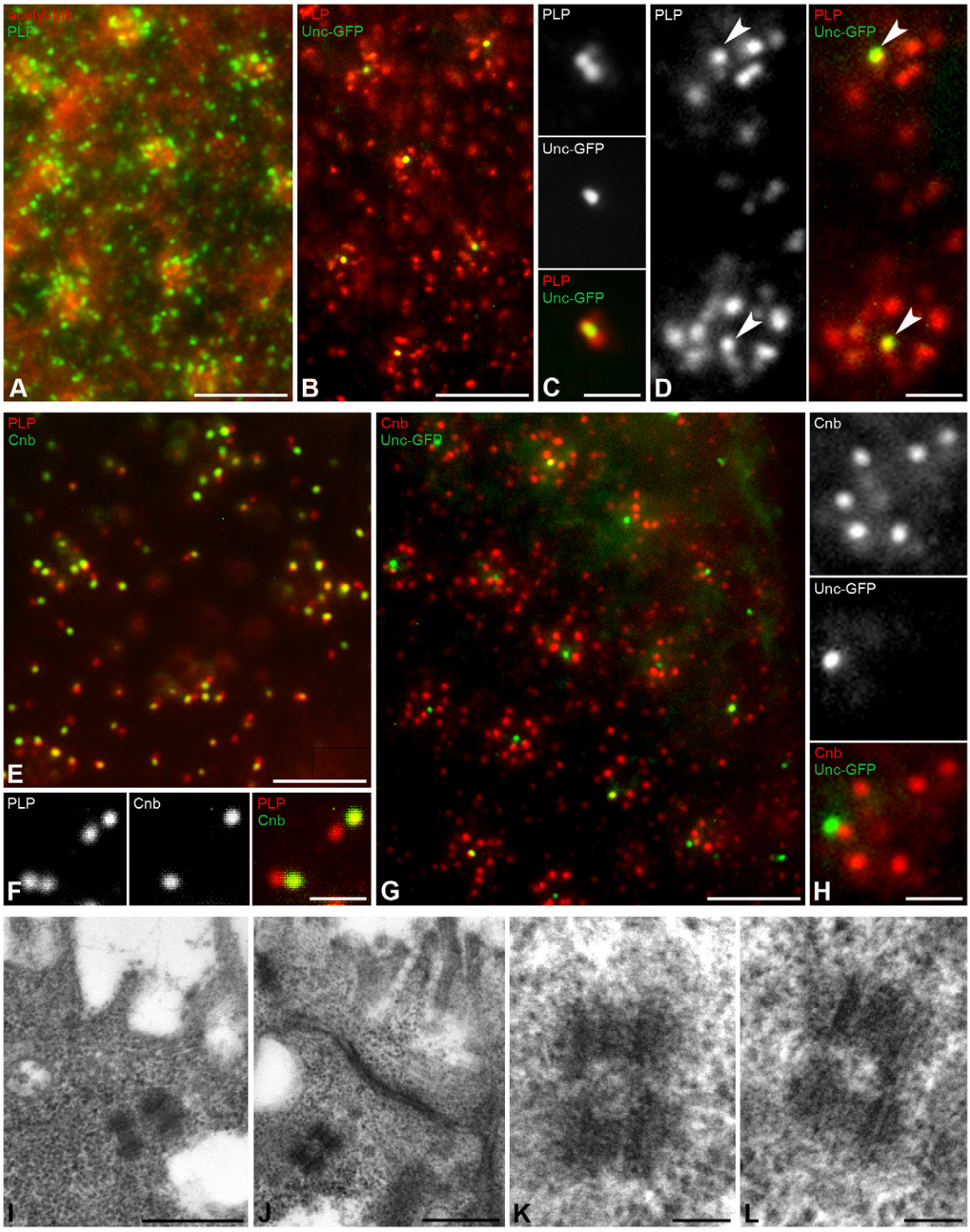

Fig. 2. Unc and mother centrioles. (A) The PLP antibody recognizes distinct spots at the apical surface of the ommatidial clusters. (B) Only one PLP spot (red) within each cluster overlaps Unc–GFP (green). (C) The Unc–GFP signal and the PLP spots partially overlap. (D) The Unc–GFP signal overlaps the more PLP-reactive centriole of the pair (arrowheads). (E) Anti-centrobin antibody (green) recognizes half of the PLP spots (red) within each ommatidial cluster. (F) The centrobin signal overlaps only one centriole of the pairs detected by PLP. (G,H) Unc–GFP (green) and centrobin (red) spots do not colocalize. (I,J) Centrioles aligned in tandem found in the apical surface of photoreceptor cells. (K,L) Details of the paired centrioles. Scale bars: 5 µm (A,B,E,G), 1 µm (C,D,F,H), 500 nm (I,J), 100 nm (K,L).

Drosophila ciliary structures (Enjolras et al., 2012). Although distinct Unc dots were evident, we did not find Chibby signals (data not shown). Therefore, the Unc-labeling we found in larval imaginal discs was restricted to the centrioles. The failure to find centrioles that interact with the plasma membrane of the photoreceptor cells (Fig. 2I–L) rules out, indeed, the possibility that these cells could have organized ciliary structures and true transition zones.

The Unc–GFP signal was no longer detected in the rhabdomeric cells of the early pupae although the centrioles were still observed with the anti-PLP antibody (not shown). Serial sections of the

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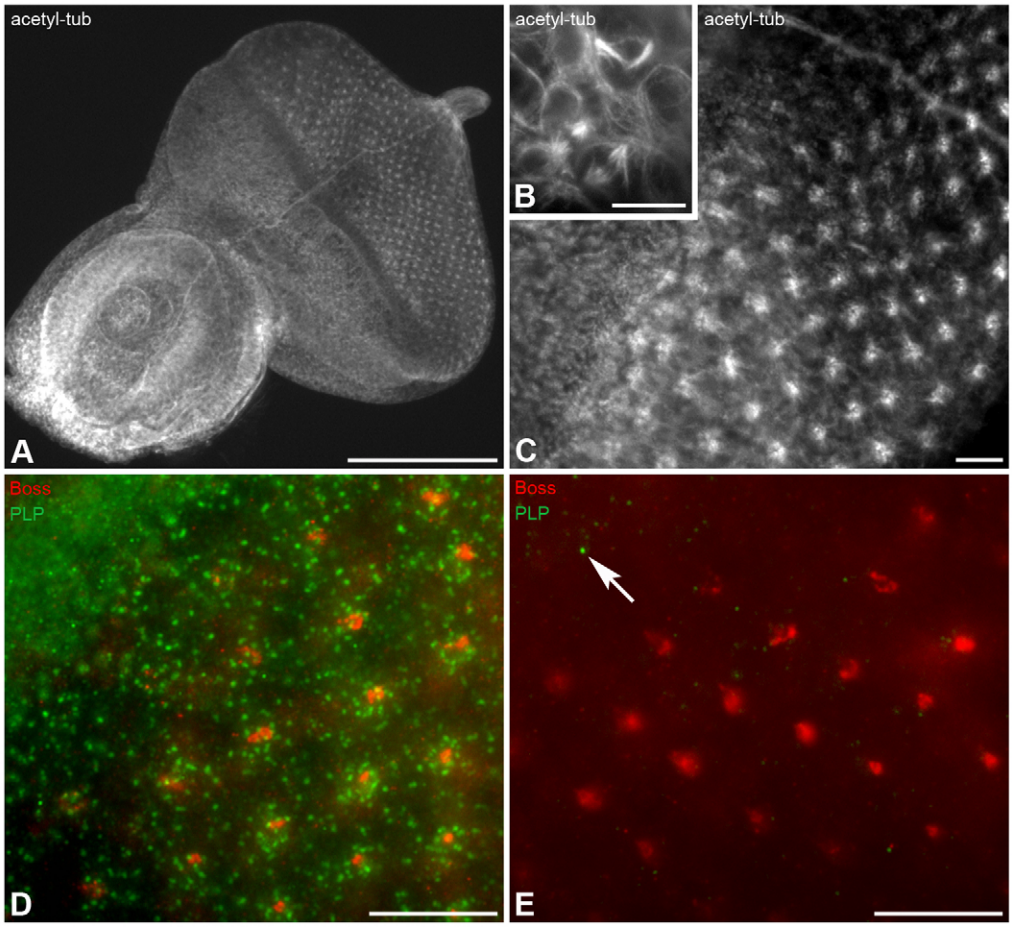

Fig. 3. Sas4 mutants assemble normal eye imaginal discs. (A) The overall organization of the eye imaginal disc looks normal in third-instar larvae. (B) The anastral mitotic spindles are assembled without centrosomes. (C) Detail showing the evenly arranged ommatidia that are characterized by an apical concentration of acetylated tubulin. (D) Control imaginal discs displaying distinct centriole clusters (green) overlapping the Boss signal (red). (E) The Boss signal (red) is present in mutant Sas-4 ommatidia, but centrioles are very rare (green, arrow). Anterior is to the left. Scale bars: 100 µm (A), 10 µm (C,D,E), 5 µm (B).

ommatidia (n=27) failed to reveal centrioles aligned in tandem (not shown), suggesting that their presence is directly correlated with the Unc–GFP signal.

Taken together these observations suggest that the Unc–GFP signal associated with mother centrioles in larval imaginal discs is not involved in axoneme organization, challenging the common view that the Unc expression is correlated with the process of centriole-to-basal body conversion.

To assess the functional significance of Unc, we examined the eye imaginal disc in *Sas4* third-instar larvae, which develop in the absence of centrioles (Basto et al., 2006). In agreement with previous observations (Basto et al., 2006), we found that *Sas4* mutants assembled properly structured eye imaginal discs (Fig. 3A) in which the ommatidial clusters were evenly spaced (Fig. 3B) and the R8 cells were clearly marked by the anti-Boss antibody. Cell proliferation was supported by anastral mitotic spindles that organize without centrosomes (Fig. 3C). Thus, the centrioles are dispensable for ommatidia development.

Given that there are some remaining centrioles in the adult tissues of Sas-4 (Basto et al., 2006), we asked whether there were centriole remnants in mutant eye imaginal discs and whether they expressed Unc–GFP. Control imaginal discs had many centrioles disposed in distinct clusters (Fig. 3D). By contrast, of 43 larval imaginal discs examined from a Sas-4 strain containing the Unc–GFP transgene only seven of them had two or three centrioles in the posterior region. However, these rare centrioles neither expressed Unc–GFP, nor co-localized with the Boss staining (Fig. 3E). The few centrioles we observed with the anti-PLP antibody in testes from the Sas-4 stock containing the Unc–GFP transgene also lacked Unc signal (not shown). The failure to find Unc–GFP agrees with previous observations showing that spermatocytes of the *Sas-4* mutants lack distinct cilium-like structures. The few centrioles observed did not grow properly and failed to migrate to the cell surface (Riparbelli and Callaini, 2011).

However if the centrioles expressing Unc–GFP do not reflect a functional significance, what they do in the rhabdomeric eye of *Drosophila*? A simple explanation could be that the Unc protein might be an earlier marker for centrioles fated to become basal bodies but unable to nucleate a true ciliary axoneme. These centrioles might represent the remnants of ciliary structures originally present in the fly ancestors. Rudimentary cilia have indeed been found in the ommatidia of other insect species (Home, 1972; Wachmann and Hennig, 1974; Wachmann et al., 1983). However, all the photoreceptor cells shaping the insect ommatidia examined to date have centrioles arranged in tandem with rudimentary ciliary projections and distinct rootlets. By contrast, only one cell in each *Drosophila* developing ommatidium displays aligned centriole pairs, without ciliary axonemes or rootlets. Given that serial sectioning showed that this unique cell was positioned in the posterior region of each developing ommatidium it is believable that this cell might be the cell expressing Unc–GFP and identified as R8 by localization of the Boss ligand. Therefore, R8, which is the founder cell for each ommatidium and promotes the sequential differentiation of the other photoreceptor cells (Tomlinson and Ready, 1987; Morante et al., 2007; Roignant and Treisman, 2009), also reflects the conservation of an ancestral ciliary type program. Interestingly, the Unc–GFP signal was found before the Boss ligand appeared, suggesting that Unc is expressed at earlier stages of R8 specification.

Thus, the *Drosophila* eye could recapitulate the evolutionary trend of the insect eye: loss of the ciliary structures, but maintenance of centrioles aligned in tandem with the distal centriole fated to become a basal body, but unable to nucleate a ciliary axoneme. These findings are consistent with a monophyletic origin of the photoreceptor cells from an ciliated ancestor cell that might have acquired surface expansions to accommodate photoreceptor pigments. This process might have been realized with the formation of microvillus-like expansions by the apical cell surface (rhabdomeric model) or by lateral finger-like extension of the ciliary membrane (ciliary model) (for a review, see Lamb, 2013).

MATERIALS AND METHODS

Drosophila strains

The stock containing the Unc–GFP transgene (Baker et al., 2004) was provided by Maurice J. Kernan (Department of Neurobiology and Behavior and Center for Developmental Genetics, Stony Brook University, Stony Brook, NY). The Sas-4 mutant allele (Basto et al., 2006) was a gift of Jordan Raff (Sir William Dunn School of Pathology, University of Oxford, UK). The unpublished fly stockes carrying Unc–mkate2 and Chibby–GFP were kindly provided by Bénédicte Durand (Institut NeuroMyoGène, Lyon, France). The Sas-4 stock containing the Unc–GFP transgene was a gift of Silvia Bonaccorsi (Dipartimento di Biologia e Biotecnologie, Sapienza Università di Roma, Rome, Italy). Flies were raised on a standard *Drosophila* medium at 24°C.

Antibodies

We used the following antibodies: chicken anti-PLP (1:500; Rodrigues-Martins et al., 2007); mouse anti-Boss (1:500; Krämer et al., 1991); rabbit anti-centrobin (1:200; catalog no. HPA023319, Sigma-Aldrich); and mouse anti-acetylated tubulin (1:100; catalog no. T6793, Sigma-Aldrich). The secondary antibodies used (1:800) were Alexa Fluor-488- and Alexa Fluor-555-conjugated anti-mouse-IgG, anti-rabbit-IgG, anti-chicken-IgG and were obtained from Invitrogen.

Immunofluorescence preparations

Eye imaginal discs from wandering third-instar larvae and white pupae were dissected in phosphate-buffered saline (PBS) and fixed in cold methanol for 10 min at −20°C or paraformaldehyde 4% in PBS for 15 min at room temperature. For antigen localization, the samples were washed for 15 min in PBS and incubated for 1 h in PBS containing 0.1% bovine serum albumin (PBS-BSA) to block non specific staining. The samples were incubated overnight at 4°C with the specific antisera in a humid chamber. After washing in PBS-BSA, the samples were incubated for 1 h at room temperature with the appropriate secondary antibodies. In all cases DNA was visualized after an incubation of 3–4 min in Hoechst 33258. Imaginal discs were mounted in small drops of 90% glycerol in PBS. Testes dissected from pupae of the Sas-4 stock expressing Unc–GFP were fixed as previously described (Riparbelli and Callaini, 2011), and stained with the anti-PLP antibody to visualize remnant centrioles. Images were taken with an Axio Imager Z1 microscope (Carl Zeiss), using an 100× objective, equipped with an AxioCam HR cooled charge-coupled camera (Carl Zeiss). Grayscale digital images were collected separately and then pseudocolored and merged using Adobe Photoshop 7.0 software (Adobe Systems).

Transmission electron microscopy

Eye imaginal discs from wandering third-instar larvae and white pupae were fixed in 2.5% glutaraldehyde buffered in PBS overnight at 4°C. After rinsing for 30 min in PBS, the samples were post-fixed in 1% osmium tetroxide in PBS for 2 h at room temperature. Samples were then washed in the same buffer, dehydrated in a graded series of ethanol, embedded in a mixture of Epon-Araldite, and polymerized at 60°C for 48 h. The serial section analysis just posterior to the morphogenetic furrow was performed by cutting the ommatidia through their apical region or along their longitudinal axis with a Reichert Ultracut E ultramicrotome equipped with a diamond knife. A series of 40–45 consecutive sections (50–60-nm thick) were collected in strips of seven or eight each on formvar-coated single slot copper grids. Sections were then stained with uranyl acetate and lead citrate. Transmission electron microscopy observations were performed with a FEI Tecnai G2 Spirit transmission electron microscope operating at an accelerating voltage of 100 kV and equipped with a Morada CCD camera (Olympus).

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Competing interests

The authors declare no competing or financial interests.

Author contributions

M.G., M.G.R. and G.C. conceived the project. M.G. and M.G.R. performed experiments. G.C. wrote the manuscript.

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