AP-1 clathrin adaptor and CG8538/Aftiphilin are involved in Notch signaling during eye development in *Drosophila melanogaster*

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Summary

Clathrin adaptor protein complex-1 (AP-1) and its accessory proteins play a role in the sorting of integral membrane proteins at the *trans*-Golgi network and endosomes. Their physiological functions in complex organisms, however, are not fully understood. In this study, we found that CG8538p, an uncharacterized *Drosophila* protein, shares significant structural and functional characteristics with Aftiphilin, a mammalian AP-1 accessory protein. The *Drosophila* Aftiphilin was shown to interact directly with the ear domain of γ-adaptin of *Drosophila* AP-1, but not with the GAE domain of *Drosophila* GGA. In S2 cells, *Drosophila* Aftiphilin and AP-1 formed a complex and colocalized at the Golgi compartment. Moreover, tissue-specific depletion of AP-1 or Aftiphilin in the developing eyes resulted in a disordered alignment of photoreceptor neurons in larval stage and roughened eyes with aberrant ommatidia in adult flies. Furthermore, AP-1-depleted photoreceptor neurons showed an intracellular accumulation of a Notch regulator, Scabrous, and downregulation of Notch by promoting its degradation in the lysosomes. These results suggest that AP-1 and Aftiphilin are cooperatively involved in the intracellular trafficking of Notch during eye development in *Drosophila*.

Key words: AP-1, Clathrin, GGA, Drosophila, Notch, TGN, Endosomes, Trafficking

Introduction

Intracellular transport of integral membrane proteins and secretion of soluble proteins both require formation of membrane-bound transport carriers in cells. A series of cytoplasmic 'coat' proteins play essential roles in the physical formation of the transport carriers and sorting of the cargo molecules into such carriers. These coat proteins are recruited onto the site of transport carrier formation by means of their specific adaptor proteins. In the transport pathways between the *trans*-Golgi network (TGN) and endosomes or lysosomes, clathrin serves as a major coat protein. Recruitment of clathrin onto those compartments for formation of the clathrin-coated vesicles (CCVs) requires clathrin adaptors such as AP-1 (adaptor protein complex-1) and GGA (Golgi-localized, γ-adaptin ear domain-containing, Arf1 binding protein) that are conserved from yeast to mammals (Robinson, 2004; Hirst et al., 2009; Kametaka et al., 2010).

AP-1 is a heterotetrameric protein complex composed of two large subunits γ (AP1γ) and β1 (AP1β1), one medium subunit μ1 (AP1μ1) and one small subunit σ1 (AP1σ1). The AP-1 complex is recruited to the TGN membrane through interaction with the membrane-bound, active forms of class I Arf small GTPases and with membrane phospholipids such as phosphatidylinositol 4-phosphate. GGA and the membrane-targeted AP-1 are believed to recognize their specific cargo molecules and link them with clathrin triskelion for concentration of the cargo at CCV budding sites to mediate formation of CCVs (Robinson, 2004).

The C-terminal regions of the large subunits of AP-1 or GGA form globular domains called 'ear' domains responsible for binding of a cohort of accessory proteins including rabaptin-5 (Hirst et al., 2000; Doray and Kornfeld, 2001; Shiba et al., 2002; Mattera et al., 2003), γ-synergin (Page et al., 1999; Hirst et al., 2000; Takatsu et al., 2000), p56 (Lui et al., 2003; Mardones et al., 2007), NECAP1 and NECAP2 (Ritter et al., 2003; Mattera et al., 2004), Aftiphilin (Mattera et al., 2004), γ-BAR (Neubrand et al., 2005), enthoprotin/epsinR/Clint (Kalthoff et al., 2002; Wasiak et al., 2002; Hirst et al., 2003; Mills et al., 2003), and liquid facets-Related (Lee et al., 2009). Recent biochemical analyses revealed that the accessory molecules interact with the γ-adaptin ear domain (hereafter referred to as γ-ear) of AP-1 or the ear domain (GAE domain) of GGA through a canonical tetrapeptide motif ΨG[PDE][ΨLM] (where Ψ is an aromatic residue) in mammals (Mattera et al., 2004). Although their precise molecular functions are still unclear, some of the accessory proteins play significant roles in intracellular protein trafficking, presumably together with the adaptor proteins (Hirst et al., 2003; Kametaka et al., 2007; Mardones et al., 2007).

In contrast to the accumulating knowledge on the molecular functions of AP-1 in cultured cells and in vitro, only limited information concerning the physiological consequences of AP-1

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has been reported. Recent analysis revealed that mutations in the human *AP1S2* gene encoding the σ2 subunit of the human AP-1 complex are associated with syndromic X-linked mental retardation, with hydrocephalus and calcifications in basal ganglia (Tarpey et al., 2006; Saillour et al., 2007). In addition, gene disruption or knockdown of AP-1 subunits in model animals including mouse and *Caenorhabditis elegans* resulted in embryonic lethality, emphasizing their functional importance in vivo (Zizioli et al., 1999; Meyer et al., 2000; Montpetit et al., 2008). As for *Drosophila,* another well-known model animal, our group and that of Hirst have previously shown that the *Drosophila* AP-1 complex and GGA function in the formation of CCVs, and help to sort LERP (*Drosophila* ortholog of mammalian cation-independent mannose 6-phosphate receptor) (Dennes et al., 2005) at the TGN in S2 cells (Hirst et al., 2009; Kametaka et al., 2010).

Eye development in the fly is initiated with selection of the first photoreceptor neuronal cells (R8 cells) from the proneuronal cell clusters at the morphogenetic furrows of the larval eye imaginal discs (Dokucu et al., 1996). In this process, the cell specification requires an intercellular signal transduction system called lateral inhibition by which neuronal cells repress development of cells in the vicinity. Several signaling molecules including Notch, Delta and Scabrous are known to be essential for the specification of R8 neuronal cells in the developing eye in *Drosophila* (Roignant and Treisman, 2009). Recently, a mutant screening in fly revealed that mutations in AP47, a μ1-subunit of AP-1, resulted in typical loss-of-function phenotypes of Notch under certain genetic backgrounds (Mahoney et al., 2006). More recently, while the current study was under preparation, additional direct evidence was reported that *Drosophila* AP-1 is involved in the trafficking of Notch at sensory organs in the notum of the fly (Benhra et al., 2011). However, the physiological functions of *Drosophila* AP-1 in other tissues remain to be elucidated.

Here, we show that *Drosophila* AP-1 plays a crucial role in the development of compound eyes in the fly. We first identified an uncharacterized ORF CG8538 as a *Drosophila* ortholog of a mammalian AP-1 accessory protein, Aftiphilin, and then showed that *Drosophila* AP-1 and CG8538p/Aftiphilin cooperatively function in the development of photoreceptor cells at the early stage of eye development. Moreover, our data raise the possibility that *Drosophila* AP-1 and Aftiphilin are involved in the Notch signaling required for the specification of photoreceptor neurons in vivo.

Results

Drosophila CG8538 encodes an Aftiphilin-related protein

Previously, Hirst and colleagues reported that a short region of approximately 70 amino acids of human Aftiphilin is well conserved over several species including the fruit fly, *Drosophila melanogaster* (Fig. 1A) (Hirst et al., 2005). Indeed, the deduced amino acid sequence of CG8538, an uncharacterized *Drosophila* ORF contains an amino acid sequence that shows a limited, but significant, homology with this short region (data not shown) (Hirst et al., 2005). In addition to this conserved region, we found several ΨG[PDE][ΨLM]-like motifs in CG8538 such as FxxΦ (where Φ is a hydrophobic residue) and WxxF motifs that could be involved in the interaction with γ-ear domains of AP-1 and GGA, and the α-adaptin ear domain of AP-2, respectively (Fig. 1B) (Duncan and Payne, 2003; Mattera et al., 2004; Burman et al., 2005). As depicted in Fig. 1A, these characteristics are found in both human Aftiphilin and *Drosophila* CG8538; thus, CG8538 was presumed to be a structural ortholog of mammalian Aftiphilin and further characterization of the gene product was carried out.

CG8538p/Aftiphilin specifically binds to the ear domain of *Drosophila* γ-adaptin

To see whether CG8538p is capable of binding to GGA or γ-adaptin of the *Drosophila* AP-1 complex, a pull-down assay was performed using the γ-ear domains (GAE domain) of *Drosophila* GGA or γ-adaptin fused with GST. As shown in Fig. 1D, the V5-tagged CG8538p expressed in S2 cells was successfully pulled-down with GST–γ-ear, but not with GST–GGA-GAE. To confirm the data and further analyze the direct interaction between CG8538p and γ-ear, a yeast two-hybrid assay was carried out. CG8538p was found to interact with mammalian γ-ears and GGA-GAEs, as well as with *Drosophila* γ-ear (Fig. 1E). In this experiment, again CG8538p failed to interact with *Drosophila* GGA-GAE, indicating that CG8538p specifically binds to the ear domain of γ-adaptin, but not to GGA-GAE.

Previous structural analyses of the γ-ear domains of mammalian adaptor molecules revealed the electrostatic interaction with their accessory molecules through a series of conserved surface amino acid residues in the γ-ear domains (Nogi et al., 2002; Kent et al., 2002) (Fig. 1C, boxed amino acid residues). The alignment of the sequences of *Drosophila* and mammalian γ-ear and GAE domains, however, indicated that most of these basic residues on the surface of the mammalian γ-ear domains are conserved in *Drosophila* γ-ear, whereas these residues are not conserved in the GAE region of *Drosophila* GGA, as mentioned previously (Kametaka et al., 2010). To see if these residues in the *Drosophila* γ-ear domain contribute to the molecular interaction with CG8538p, amino acid substitutions A910Q, R948A, R950A or R952A (Fig. 1C, asterisks) were introduced to the *Drosophila* γ-ear domain, and their binding capacity to CG8538p was examined by yeast two-hybrid assay. With any substitution, the interaction was reduced drastically, indicating that the conserved surface residues in the *Drosophila* γ-ear domain are crucial for interaction with CG8538 (Fig. 1F). It is most likely that these residues in the γ-ear domain serve as a bona fide platform for the interaction with CG8538p, as is the case for mammalian γ-ear, and that *Drosophila* GGA fails to interact with CG8538 because it lacks these residues (Fig. 1C).

Next, to identify the region of CG8538p that is responsible for the interaction with γ-adaptin, a yeast two-hybrid experiment was performed using a series of N-terminal truncation forms of CG8538p (Fig. 1A,G). The interaction was dramatically reduced when the construct lacked the region 301–450, which includes five out of eight putative γ-ear binding motifs of CG8538p (Fig. 1A) suggesting that the N-terminal 301–450 region of CG8538p is responsible for the interaction with the γ-ear domain. These results indicate that CG8538p shares significant characteristics with mammalian Aftiphilin, i.e. it contains multiple γ-ear binding motifs and is able to interact with γ-adaptin in vitro, in addition to the limited but significant sequence similarity. Thus, we hereafter designate CG8538 as *Drosophila* Aftiphilin.

Drosophila Aftiphilin is localized to the *trans*-Golgi together with AP-1 in S2 cells

In mammals, Aftiphilin is localized to the TGN membrane through an interaction with the γ-ear domain of AP-1 (Hirst et al.,

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2005). To see whether this is also the case for *Drosophila* Aftiphilin, the C-terminally V5-tagged *Drosophila* Aftiphilin was expressed in S2 cells or HeLa cells, and the intracellular localization was analyzed by immunofluorescence microscopy. *Drosophila* Aftiphilin was mainly localized to the punctate structures adjacent to, but not coincident with, the *cis*-Golgi marker dGM130 or *medial*-Golgi marker p120, and it was preferentially colocalized with γ-adaptin, suggesting that *Drosophila* Aftiphilin associates with the *trans* side of the Golgi complex (Fig. 2A). Moreover, Aftiphilin was also localized at more fine structures at the cell periphery (Fig. 2A, arrowheads; see Discussion). When expressed in HeLa cells, *Drosophila* Aftiphilin-V5 was localized at the perinuclear Golgi area, and it overlapped with γ-adaptin, suggesting its localization at the TGN (see supplementary material Fig. S1).

Next, to test whether *Drosophila* Aftiphilin associates with the Golgi in an Arf1-dependent manner, as has been observed for mammalian AP-1, S2 cells were treated with a fungal toxin BFA that causes *Drosophila* ARF1/Arf79F small GTPase to dissociate from Golgi compartments. To detect the endogenous Aftiphilin, we generated a specific antibody to *Drosophila* Aftiphilin (Fig. 2B). As shown in Fig. 2C, endogenous Aftiphilin was localized at the intracellular organelles including Golgi compartments (like the V5-tagged Aftiphilin shown in Fig. 2A) and was dissociated from the Golgi membrane by treatment of the cells with 10 μg/ml BFA for 10 minutes. γ-adaptin was also dissociated from the Golgi membrane by using the same treatment (Fig. 2C). This result suggests that *Drosophila* Aftiphilin is associated with the Golgi membrane in an Arf1-dependent manner, as in mammals.

***Drosophila* Aftiphilin interacts with AP-1 and AP-2 complexes**

To further confirm the interaction between *Drosophila* Aftiphilin and AP-1 in vivo, immunoprecipitation experiments were performed. V5-tagged μ-subunits of *Drosophila* AP-1 (AP1μ1-V5), AP-2 (AP1μ2-V5) or AP-3 (AP1μ3-V5), or V5-tagged Aftiphilin stably expressed in S2 cells were immunoprecipitated with anti-V5 antibody and the precipitated proteins were analyzed by immunoblotting. Endogenous *Drosophila* Aftiphilin was coimmunoprecipitated with AP1μ1-V5 (Fig. 3B), and the endogenous AP1γ subunit was also co-precipitated with Aftiphilin-V5 (Fig. 3C), strongly suggesting that Aftiphilin forms a complex with AP-1 in vivo. Interestingly, a very small but significant amount of Aftiphilin was co-precipitated with AP2μ2-V5 (Fig. 3B) and vice versa (see supplementary material Fig. S2), suggesting a weak interaction between *Drosophila* Aftiphilin and AP-2 complex. As expected from the result of the yeast two-hybrid experiment in Fig. 1, *Drosophila* GGA was not co-precipitated with Aftiphilin-V5, but with APμ1-V5 (Fig. 3D; see Discussion). Taken together, these results suggest that, in S2 cells, *Drosophila* Aftiphilin specifically forms a complex with AP-1 and possibly with AP-2, but not with GGA.

Depletion of *Drosophila* AP-1 or Aftiphilin causes defects in eye development in adult flies

Previous studies on the physiological functions of AP-1 revealed that depletion of an AP-1 component causes lethality in mammals, zebrafish, worms and flies (Zizioli et al., 1999; Meyer et al., 2000; Shim et al., 2000; Zizioli et al., 2010; Benhra et al., 2011; Burgess et al., 2005). Because we were interested in how the Golgi clathrin adapter machineries are involved in the development of specific organs of the fly, AP-1 components or other related genes were depleted in the eyes using the GAL4-dependent knockdown system (Dietzl et al., 2007), and phenotypic analysis of the knockdown flies was carried out.

First, UAS-IR (inverted repeat) lines for each subunit of *Drosophila* AP-1 complex or other related genes including those encoding Aftiphilin, GGA, clathrin heavy chain (CHC) and Arfs were crossed with *actin*-GAL4 driver to validate the RNA interference (RNAi) system. As shown in Table 1, depletion of these genes with the tissue-nonspecific and constitutive driver caused death during development in larval or pupal stages, suggesting that these silencing constructs work properly and that these genes have crucial functions during normal development of the fly. By contrast, crossing with the *GMR*-GAL4 driver, whose expression is restricted to the late stage of compound eye development, showed a series of genes as susceptible. These genes, encoding Arf79F, Arf102F, BAP1 and CHC, are supposed to be required for maintenance of cell viability. When *eyeless* (*ey*)-GAL4, whose expression is induced from the early larval stages in the central nervous system and eye antennal primordium, was used for gene silencing, we found that RNAi of *Drosophila* AP-1 subunits, including AP1σ1, AP1γ and AP47 (AP1μ1), or of Aftiphilin resulted in roughened eyes with irregular alignment of ommatidia and bristles, whereas no significant phenotype was seen in the case of *Drosophila* GGA or LERP knockdown (Table 1, Fig. 4A,B,D). These results suggest that *Drosophila* AP-1 and its accessory molecule Aftiphilin specifically play an important role during eye development. Similar results were obtained with at least two different RNAi constructs with distinct target sequences, confirming that the observed phenotype is gene-specific (Table 1). In addition, we noticed that RNAi of *Drosophila* Aftiphilin tends to cause smaller eyes with decreased number of ommatidia (see supplementary material Fig. S3), suggesting that Aftiphilin is also involved in the cell proliferation during eye development (see Discussion).

In addition to the RNAi experiments, we also performed mosaic analysis to examine the cells mutant for a loss-of-function allele of AP1μ1 mutation [$AP47^{SHE11}$ (Mahoney et al., 2006; Benhra et al., 2011)], for the eye phenotype. Ommatidia with homozygous $AP47^{SHE11}$ allele showed roughened phenotype (Fig. 4C, arrows) similar to that of the AP-1 or Aftiphilin knockdown flies, indicating that AP-1 is required for normal eye development.

Genetic interaction between *Drosophila* AP-1 and Aftiphilin

From the molecular characterization of *Drosophila* AP-1 and Aftiphilin in S2 cells (Figs 1–3), these proteins were expected to function cooperatively in vivo. To assess this possibility, a double knockdown of AP1μ1 and Aftiphilin under control of *ey*-GAL4, was performed to examine their genetic interaction. A single knockdown of AP1μ1 reduced the number of adult flies significantly, and additional knockdown of Aftiphilin resulted in complete loss of viable adults as shown in Table 2. Detailed observations revealed that they died at the pharate adult stage in pupal periods with loss of the head (not shown). By contrast, no significant effect was seen with double knockdown of AP1μ1-GGA or AP1μ1-LERP (Table 2). These results suggest that *Drosophila* AP-1 and Aftiphilin play important roles in closely related biological processes in vivo.

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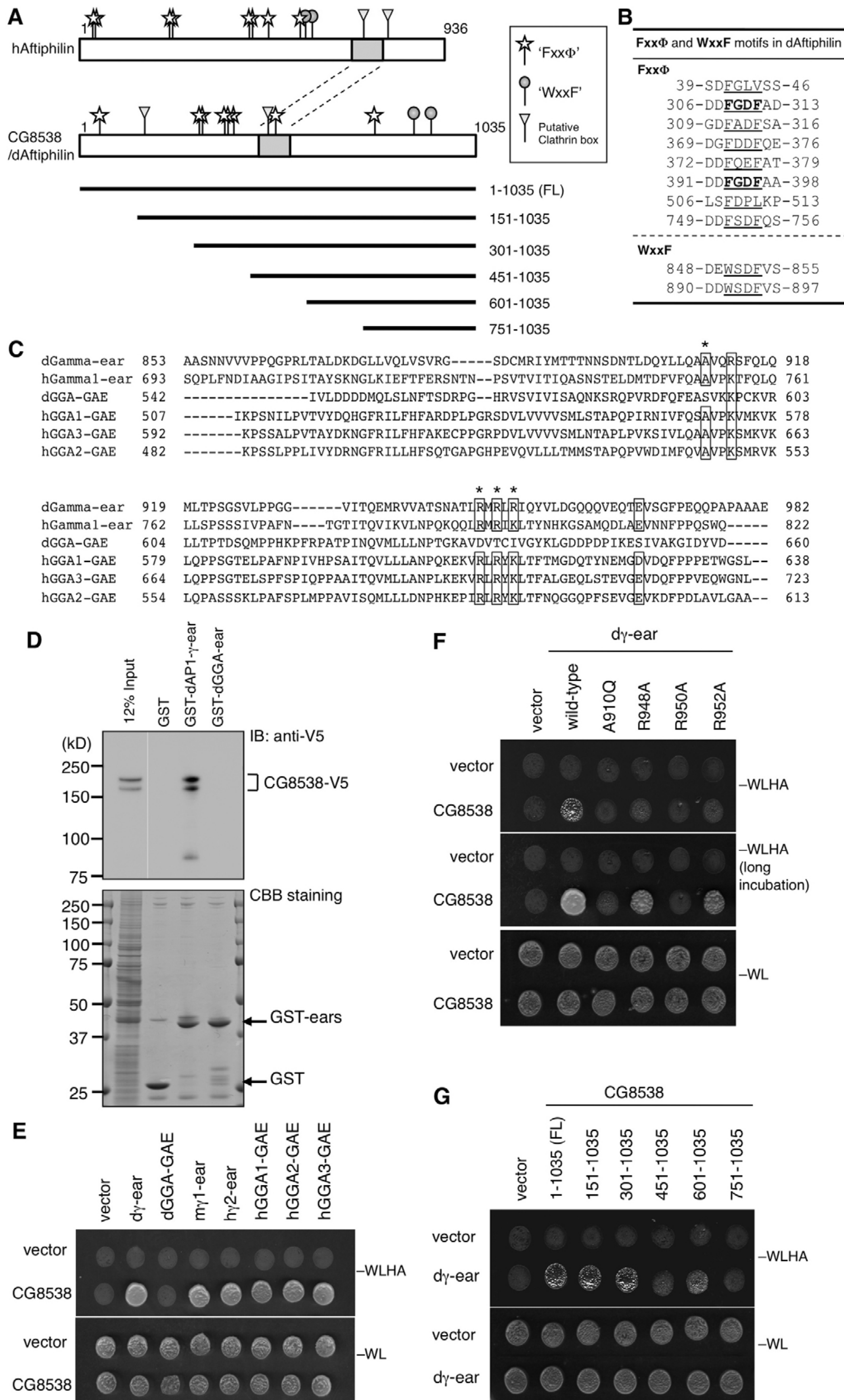

Fig. 1. See next page for legend.

RNAi of *Drosophila* AP-1 and Aftiphilin causes aberrant ommatidial structures

To further analyze the eye phenotypes found in the AP-1 or Aftiphilin knockdown flies, the morphology of the retina of adults was analyzed (Fig. 4D,E). In the wild-type retina, well-aligned and hexagonal-shaped ommatidia surrounded by pigment cells that contain pigment granules were seen (Fig. 4Da′,Db′; Fig. 4E, arrowheads), and in each ommatidium, seven Toluidine-Blue-positive rhabdomere structures derived from photoreceptor neurons R1 to R7 were observed (Fig. 4D,E). By contrast, in the eyes of AP-1 or Aftiphilin knockdown flies, irregular sizes of ommatidia with excess (Fig. 4Db–De, black arrows) or fewer (Fig. 4Db–De, white arrows) numbers of rhabdomeres were observed. Electron microscopy of the ommatidial structure revealed that each ommatidium contained normally polarized photoreceptor cells, suggesting that the morphological phenotypes in the compound eyes are due to defects in photoreceptor clustering during ommatidial formation (Fig. 4E).

Axon connectivity of photoreceptor cells to the brain is affected in AP1σ1 knockdown adult flies

Next, to determine whether depletion of *Drosophila* AP-1 affects the projection of photoreceptor axons to the adult brain, we assessed the organization of axonal projections of R cells into the medulla of the brain. The medulla is subdivided into ten layers (M1–M10) based on the terminals of the innervating afferents: the R8 and R7 axons project to the M3 and M6 layers, respectively (Ting et al., 2007). In the wild type, each R8 and R7 axon visualized with monoclonal antibody against chaoptin (24B10) terminated at the M3 and M6 layer of the medulla, respectively, where it formed a spherical terminus or a synaptic bouton, which was spatially restricted to a single column (Fig. 4F). Most of the axons from R cells in the AP-1-deficient flies appeared to terminate correctly, but some axons invaded neighboring columns (Fig. 4F, arrows) or elongated beyond the M6 layer (Fig. 4F, arrowheads). These results indicate that depletion of AP-1 caused aberrant axonal projections as well, suggesting that *Drosophila* AP-1 functions in axon targeting mechanisms.

Misalignment of photoreceptor neurons occurs during eye development in AP-1-deficient larva

Morphological analyses of the adult retina allowed us to presume that the defect in *Drosophila* AP-1 function leads to the misalignment of photoreceptor cells at the earlier stages of developing eyes. Thus, the eye imaginal discs were dissected from the third instar larva, in which the alignment of photoreceptor cells was examined by staining with Alexa-Fluor-594-conjugated phalloidin. Irregularly aligned and multiplied photoreceptor cells were seen in the eye discs of the AP-1 or Aftiphilin knockdown larva, whereas normally spaced photoreceptor cells were observed in those of the wild-type larva (Fig. 5Aa–Ac), indicating that the photoreceptor cells had already misaligned in the late stage of the larval eye development. Consistent with these results, in mosaic analysis, clones mutant for $AP47^{SHE11}$ also showed aberrant alignment of photoreceptor cells (Fig. 5Ad–Ag).

Initiation of R8 photoreceptor neuron is affected in *Drosophila* AP-1 or Aftiphilin knockdown larva

The development of photoreceptor cells is initiated after the first photoreceptor cell (the R8 photoreceptor neuron) emerges from proneuronal cluster cells at the morphogenetic furrow (MF) of the eye disc, and each R8 neuron subsequently induces adjacent neurons, including R7 neurons (Roignant and Treisman, 2009). To examine the alignment of the R8 and R7 neurons in the *Drosophila* AP-1 or Aftiphilin knockdown larva, eye discs were stained with antibodies for the R8 marker Senseless (Sens) and the R7 marker Prospero (Pros). The pattern of the emerging R8 neurons was disturbed, with unusual clusters found in the AP1σ1, AP1μ1 or Aftiphilin knockdown eye discs (Fig. 5Bh–Bj, arrows). Although the pattern of R7 neurons was also affected in the AP-1 knockdown eye discs compared with that in the wild type, each R7 neuron was present in the vicinity of an R8 neuron, suggesting that the induction of R7 neurons by R8 photoreceptor cells occurred normally (Fig. 5BAa–Af).

Notch expression is affected in the AP-1- or Aftiphilin-deficient eye disc

The initial patterning of R8 photoreceptors at the MF region is dependent on lateral inhibition between the neighboring cells through the cell surface signaling molecules Notch and Delta (Roignant and Treisman, 2009). To see whether the depletion of *Drosophila* AP-1 or Aftiphilin affects the expression of these signaling molecules, the expression level of Notch in the isolated eye discs was assessed by immunoblotting and immunofluorescence microscopy. As shown in Fig. 6A, knockdown of AP1σ1 resulted in a reduction in expression of AP1γ and full-length Notch to approximately 37% and 13%, respectively. Depletion of Aftiphilin caused a slight reduction in Notch expression to approximately 55% and a reduction in AP1γ expression to approximately 60%, suggesting that destabilization of the AP-1 complex occurred by depletion of Aftiphilin (Fig. 6A). To assess the Notch expression

Fig. 1. CG8538 encodes a protein that possesses several putative FxxΦ γ-ear binding motifs. (**A**) Representation of human Aftiphilin and *Drosophila* Aftiphilin/CG8538. The truncation constructs of CG8538 are indicated by thick lines. The conserved regions of 70 amino acid residues (Hirst et al., 2005) are indicated by grey boxes. The FxxΦ γ-ear binding motifs (stars), WxxF α-ear binding motifs (circles) and putative clathrin binding boxes (triangles) are indicated. (**B**) Putative γ-ear and α-ear binding motifs found in CG8538p are shown above and below the dashed line, respectively. The tetrapeptide motifs are underlined and motifs that match ΨG[PDE][ΨLM] are marked in bold. (**C**) Sequence comparison of the *Drosophila* γ-ear domains (dGamma-ear), human γ1 (hGamma1-ear), *Drosophila* GGA (dGGA-GAE), and human GGAs (hGGA1-GAE, hGGA2-GAE and hGGA3-GAE). Conserved amino acid residues required for interaction with accessory molecules in human γ-ears (Nogi et al., 2002; Kent et al., 2002) are boxed, and asterisks mark the mutated sites. (**D**) GST pull-down assay. Total cell lysate prepared from S2 cells expressing *Drosophila* Aftiphilin/CG8538-V5 was subjected to pull-down assay with GST, GST–γ-ear or GST–GGA-ear (lower panel). Precipitated CG8538-V5 was detected by immunoblotting using anti-V5 antibody (upper panel). (**E**) *Drosophila* Aftiphilin/CG8538 interacts with *Drosophila* γ-ear and mammalian γ-ears, but not with *Drosophila* GGA-ear. Molecular interaction between CG8538 and ear domains from *Drosophila* γ-adaptin and GGA and mouse γ1, human γ2, and human GGAs was assayed with a yeast two-hybrid system. (**F**) Mutations in the conserved surface amino acid residues (as marked in C with asterisks) in the *Drosophila* γ-ear affect the interaction with CG8538. (**G**) The N-terminal region of *Drosophila* Aftiphilin/CG8538 is important for interaction with γ-ear. The N-terminal truncated series of CG8538 (see A) were subjected to the yeast two-hybrid assay to examine their ability to bind to the γ-ear domain of AP-1.

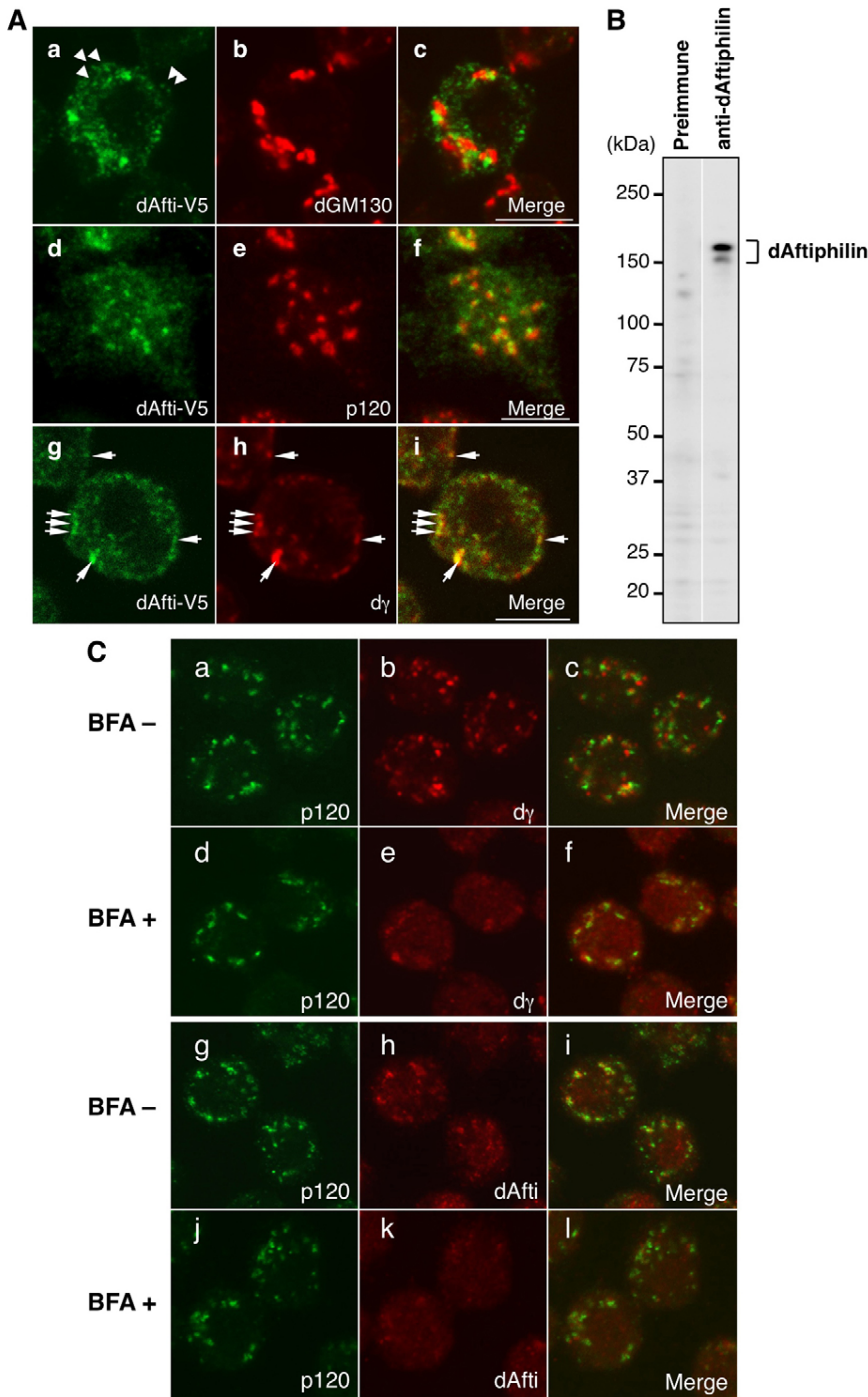

Fig. 2. *Drosophila* Aftiphilin colocalizes with AP-1 at the Golgi compartments in a BFA-sensitive manner. (**A**) S2 cells transiently expressing *Drosophila* Aftiphilin-V5 (dAfi-V5) were fixed and stained with anti-V5 antibody (green in a,c,d,f,g,i) and marker proteins (red) such as GM130 (b,c), p120 (e,f) and γ-adaptin (h,i). Arrows indicate puncta that are double-positive for Aftiphilin and γ-adaptin, and arrowheads indicate Aftiphilin-positive puncta that are associated with the peripheral structures. (**B**) Immunoblotting of endogenous *Drosophila* Aftiphilin. Whole cell lysate prepared from S2 cells was subjected to immunoblotting using pre-immune serum or the anti-Aftiphilin antibody. Bands corresponding to Aftiphilin are indicated by a parenthesis. (**C**) Dissociation of *Drosophila* Aftiphilin from the Golgi membrane by BFA treatment. S2 cells were treated with (d–f,j–l) or without (a–c,g–i) 10 μg/ml BFA for 10 minutes and then stained with anti-p120 (green in a,c,d,f,g,i,j,l) and anti-γ-adaptin (red in b,c,e,f) or anti-Aftiphilin (red in h,i,k,l). Scale bars: 5 μm.

pattern in these eye discs, immunofluorescence microscopy was carried out. Comparison of projection images that included apical and subapical intracellular regions of cells showed that overall signals, including both diffuse and punctuate signals, were significantly reduced in the AP-1 knockdown eye discs (Fig. 6Bb,Bd). Because Notch is known to be localized in intracellular vesicles and on the cell surface, especially at the adherens junction area, detailed confocal microscopy was performed. As shown in Fig. 6C, Notch signal along the DE-cadherin-positive adherens junctions area was slightly reduced (Fig. 6Cb,Ce,Cc′,Cf′), whereas there was no obvious change in the intracellular punctate signal for Notch at the middle level of the cells (Fig. 6Ch,Ck). Thus, relatively more Notch protein was distributed in the intracellular vesicles than on the cell surface in the AP-1 knockdown eye disc.

Mistargeting of Notch to the endosomal and lysosomal compartments in AP-1-deficient cells

Because the expression level of the mRNA for Notch was comparable between the control and AP-1 knockdown eye discs

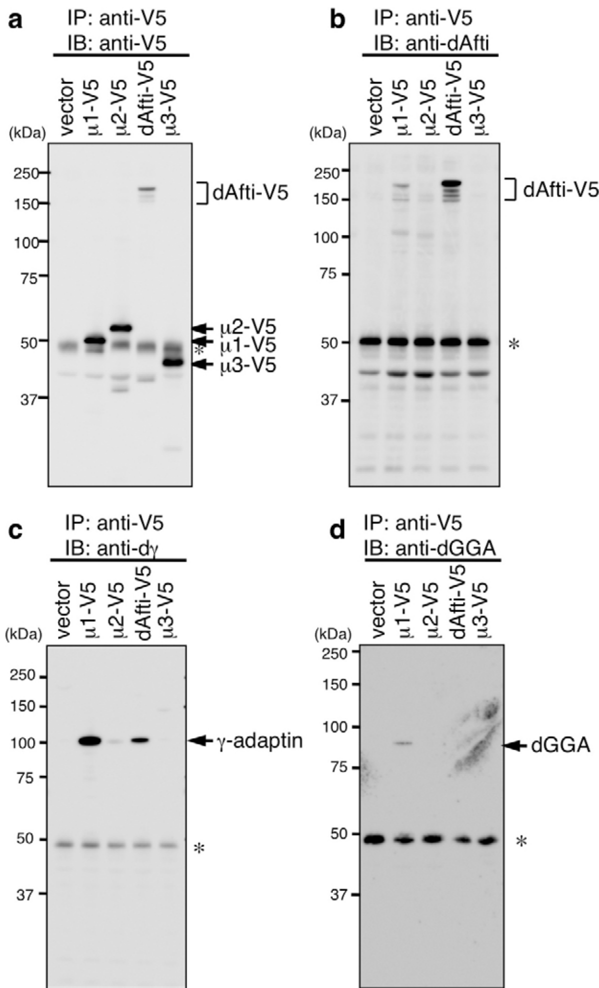

Fig. 3. ***Drosophila*** **Aftiphilin forms a stable complex with AP-1 and AP-2.** Cell lysate prepared from S2 cells transiently transfected with empty vector (vector) or from S2 cells stably expressing *Drosophila* AP1µ1-V5 (µ1-V5), AP2µ2-V5 (µ2-V5), AP3µ3-V5 (µ3-V5) or Aftiphilin-V5 (dAfti-V5) were subjected to immunoprecipitation with anti-V5 followed by immunoblotting with (a) anti-V5, (b) anti-Aftiphilin (anti-dAfti), (c) anti-AP1γ1 (anti-dγ) or (d) anti-GGA antibodies. Aftiphilin was coimmunoprecipitated with AP-1 or AP-2, but not with AP-3. Asterisks indicate the heavy chain of immunoglobulin.

(see supplementary material Fig. S4), the decrease in the cellular Notch protein was presumed to be due to its lysosomal degradation. To assess this possibility, organ culture of eye discs was carried out in the presence or absence of chloroquine, which inhibits lysosomal protein degradation (Vaccari and Bilder, 2005). After incubation of the wild-type or AP1σ1 knockdown eye antennal discs with chloroquine, Notch signal in the Rab7-positive late endosomes was significantly increased in the AP1σ1 knockdown eye discs but not so evident in the wild-type eye disks (see supplementary material Fig. S5). Because similar results were also obtained when the mosaic eye discs were cultured for 10 hours in the presence of chloroquine (Fig. 7A), we performed quantitative analysis by counting Notch/Rab7 double-positive dots using this experimental system. Although there was no significant difference in the number of the Notch/Rab7 double-positive signal between the wild-type (GFP-positive) and *AP47*^{SHE11} mutants without chloroquine treatment, it was significantly higher (approximately 2.5-fold) in the *AP47*^{SHE11} mutants than in the wild-type cells after the chloroquine treatment (Fig. 7B). As expected from the images for Rab7 (Fig. 7A), there was no significant difference in the number of Rab7-positive compartments between wild-type and *AP47*^{SHE11} mutant cells in both conditions (see supplementary material Fig. S6). These results strongly suggest that in AP-1-defective cells Notch protein tends to be missorted to the late endosomal and lysosomal compartments for degradation, which might cause the reduction in the steady-state levels of Notch in the AP-1 or Aftiphilin knockdown flies.

Intracellular accumulation of Scabrous in the AP-1 or Aftiphilin knockdown cells.

Scabrous is a glycosylated secretory protein expressed in the R8 neurons in the developing eye imaginal disc and is known to be involved in the determination of R8 fate through Notch signaling (Mlodzik et al., 1990; Lee et al., 1996). Secreted Scabrous protein interacts with the extracellular domain of Notch and can stabilize Notch at the cell surface (Powell et al., 2001). As shown in Fig. 8, accumulation of Scabrous in the intracellular fine compartments was observed in the R8 photoreceptor cells of *Drosophila* AP-1 or Aftiphilin knockdown cells (Fig. 8). The number of Scabrous-positive puncta per Sensless-positive R8 cell increased in the AP-1 or Aftiphilin knockdown cells by approximately 1.7- and 1.4-fold, respectively, compared with

Table 1. Phenotypic analysis of RNAi lines

	Target genes	*actin*-GAL4	*GMR*-GAL4	*ey*-GAL4
UAS-GFP	–	Viable	Normal	Normal
	AP47/APµ1*	Lethal (p)	Normal	Rough
	AP1γ1*	Lethal (p)	Normal	Rough
	AP1σ1*	Lethal (p)	Normal	Rough
	BAP/AP1β1/AP2β2*	Lethal	Rough	Lethal
UAS-IR	GGA*	Lethal (p)	Normal	Normal
	Aftiphilin/CG8538*	Lethal (p)	Normal	Rough
	LERP*	Viable	Normal	Normal
	Arf79F/Arf1*	Lethal	Rough	Rough
	Arf102F/Arf5	Lethal	Rough	Rough
	CHC	Lethal	Rough	Rough

*At least two independent RNAi lines from different sources were tested.
Rough, roughened eye; p, pupal stage; Normal, normal compound eye morphology.

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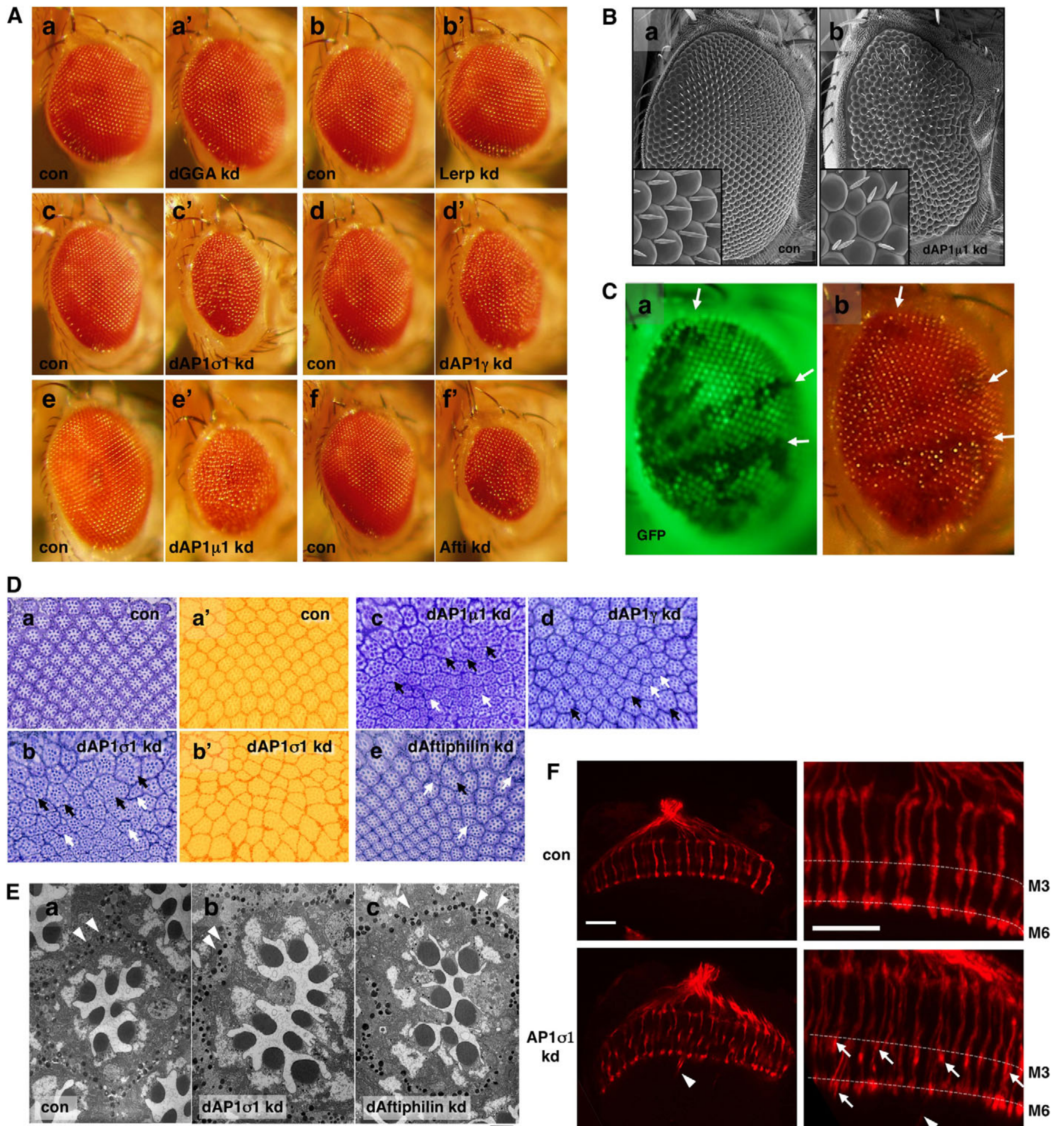

Fig. 4. Phenotypic analysis of AP-1 or Aftiphilin knockdown flies. (**A**) Homozygous UAS-IR-lines for *Drosophila* GGA (a,a′), LERP (b,b′), AP1σ1 (c,c′), AP1γ (d,d′), AP1μ1/dAP47 (e,e′) and Aftipilin (f,f′) were crossed with *ey*-GAL4/SM1 driver strain. Phenotypes of the adult compound eyes were examined in the control UAS-IR/SM1 flies (con; a–f) and UAS-IR/*ey*-GAL4 knockdown flies (kd; a′–f′). (**B**) Scanning electron microscopy of compound eyes in the control (a) and AP1μ1/AP47 knockdown (b) flies. (**C**) AP-1 depletion causes roughened eyes. To generate the mosaic fly for AP47 mutant cells, *ey*-FLP;; FRT82B *ubi*-GFP was crossed with FRT82B *AP47*^{SHE11}/TM6. Clones for *AP47*^{SHE11}*,* which are identified by the absence of GFP fluorescence (a, arrows), show roughened eye phenotype (b, arrows). (**D**) Irregular alignment of ommatidia in AP-1 or Aftiphilin knockdown flies. Alignment of ommatidia in the adult eyes from wild-type (a,a′) and from AP1σ1 (b,b′), AP1μ1/AP47 (c), AP1γ (d) and Aftiphilin (e) knockdown flies was examined by Toluidine Blue staining (a–e) to visualize the rhabdomere structures. Unstained sections are also shown (a′,b′) to detect pigment cells that contain melanin granules. Ommatidia containing excess and fewer rhabdomeres are indicated by black and white arrows, respectively. (**E**) Transmission electron microscopy of the rhabdomere structures in wild-type (a) and in AP1σ1 (b) and Aftiphilin knockdown (c) ommatidia. Arrowheads indicate melanin granules in the pigment cells. (**F**) Sagital sections of adult brains prepared from wild-type (con) or AP1σ1 knockdown flies were stained with 24B10 antibody to visualize the photoreceptor axons. Right-hand images show the M3 and M6 layers of the medulla in the left-hand images at a higher magnification. Arrows indicate axons that invaded neighboring columns. Arrowheads indicate axons elongated beyond the M6 layer. Scale bars: 10 μm.

Table 2. Genetic interaction between *Drosophila* AP1μ1 and Aftiphilin

ey-GAL4 driven genes	Genotype	Population (%)
IR{AP1μ1};{UAS-GFP} (*n*=172)	+	30.8
	AP1μ1^{-}	7.6
	UAS-GFP	29.1
	AP1μ1^{-}, *UAS-GFP*	25.0
	(Pupal death	7.6)
IR{AP1μ1};{Aftiphilin} (*n*=274)	+	36.1
	AP1μ1^{-}	4.9
	Aftiphilin^{-}	13.9
	AP1μ1^{-}, *Aftiphilin*$^{-}$	0
	(Pupal death	45.1)
IR{AP1μ1};{AP1γ} (*n*=113)	+	38.1
	AP1μ1^{-}	6.2
	AP1γ^{-}	
	AP1μ1^{-}, *AP1γ*$^{-}$	19.5
	(Pupal death	15.9)
IR{AP1μ1};{GGA} (*n*=50)	+	34.0
	AP1μ1^{-}	8.0
	GGA^{-}	40.0
	AP1μ1^{-}, *GGA*$^{-}$	8.0
	(Pupal death	10.0)
IR{AP1μ1};{LERP} (*n*=147)	+	25.2
	AP1μ1^{-}	4.1
	LERP^{-}	30.6
	AP1μ1^{-}, *LERP*$^{-}$	24.5
	(Pupal death	15.7)

IR{AP1μ1}/SM1; IR{X}/TM3 was crossed with *ey*-GAL4/*ey*-GAL4; +/+ flies, and the number of offspring counted.

those in the control cells (Fig. 8m). Moreover, there was no significant difference in the cell surface staining of Scabrous between wild-type and AP47-defective cells (see supplementary material Fig. S7). These results suggest that *Drosophila* AP-1 is involved in the intracellular trafficking of Scabrous.

Discussion

AP-1 and GGAs are the major clathrin adaptors that function at the post-Golgi compartments in species ranging from yeast to mammals. After a decade of biochemical and cell biological approaches, however, functional specificity of each adaptor at a molecular level still remains to be solved. In the present study, we showed that *Drosophila* AP-1 and its novel accessory protein Aftiphilin, but not GGA, are required for eye development, suggesting that the *Drosophila* AP-1–Aftiphilin protein complex is involved in the intracellular trafficking of specific cargo molecule(s) distinct from those regulated by GGA during eye development. We previously reported that the GAE domain of *Drosophila* GGA lacks major conserved amino acid residues potentially required for interaction with the accessory molecules that possess the tetrapeptide ΨG[PDE][ΨLM] motif (Mattera et al., 2004; Kametaka et al., 2007; Kametaka et al., 2010). Consistent with this, we showed in the current study that *Drosophila* GGA failed to interact with Aftiphilin, suggesting that the GAE domain of GGA is not structurally conserved. This finding might also reflect the physiological functional diversity between *Drosophila* AP-1 and GGA. However, the interaction between AP-1 and GGA was detected in the coimmunoprecipitation analysis (Fig. 3D), thus *Drosophila* AP-1 might also have a certain functional mode to form a complex with GGA, as implicated in mammalian cells (Bai et al., 2004).

Drosophila Aftiphilin is a physiological counterpart of mammalian Aftiphilin

In a previous study, Hirst and coworkers suggested that CG8538, an ORF in the *Drosophila* genome, encodes a protein with a limited homology with human Aftiphilin (Hirst et al., 2005). We concluded that *Drosophila* Aftiphilin/CG8538p is a functional counterpart of mammalian Aftiphilin, because of their common characteristics such as the possession of multiple γ-ear binding motifs, specific interaction with the γ-ear of AP-1, and the colocalization with AP-1 at the *trans*-Golgi compartments. Interestingly, the molecular basis of the interaction between Aftiphilin and the γ-adaptin of the AP-1 complex was also well conserved over species, because ectopically expressed *Drosophila* Aftiphilin in HeLa cells was also colocalized with γ1-adaptin of AP-1 (see supplementary material Fig. S1). Thus, the results indicate that *Drosophila* could serve a good model system to dissect the molecular mechanisms of AP-1 and Aftiphilin functions.

In the deduced amino acid sequence of *Drosophila* Aftiphilin/CG8538p, two WxxF-type binding motifs for the α-subunit of AP-2 complex were found (Fig. 1). In mammals, Aftiphilin was shown to interact with AP-1 and AP-2, and was also proposed to function with AP-2 at the endocytic pathway in neuronal cells (Burman et al., 2005). In S2 cells, *Drosophila* Aftiphilin is predominantly associated with AP-1-positive Golgi compartments and forms a stable complex with AP-1. Moreover, we could detect the molecular interaction between *Drosophila* Aftiphilin and AP-2 (Fig. 3B, supplementary material Fig. S2). Although the interaction seems to be minor compared with the interaction with AP-1, it is likely that Aftiphilin has other functions that are not related to AP-1, because the Aftiphilin-depleted fly occasionally showed much smaller eyes with decreased number of ommatidia in addition to the roughened eye phenotype (see supplementary material Fig. S3b'). Precise analysis of the physiological functions of *Drosophila* Aftiphilin is ongoing.

Drosophila AP-1 is involved in Notch signaling during eye development

Eye-specific depletion of *Drosophila* Aftiphilin or of any of the σ1- or μ1-subunits of AP-1 caused misalignment of the photoreceptor neurons due to generation of extra R8 neurons during eye development. A genetic screening for Notch modifier genes suggested that *AP47*, which encodes the μ1 subunit of *Drosophila* AP-1, is involved in Notch signaling (Mahoney et al., 2006). Another genome-wide RNAi screening showed that the subunits of *Drosophila* AP-1 and Aftiphilin/CG8538 are involved in Notch signaling (Mummery-Widmer et al., 2009). Recently, Benhra and coworkers also reported that *Drosophila* AP-1 depletion led to mislocalization of Notch and its regulator Sanpodo (Spdo) to the apical plasma membrane and the adherens junction in the sensory organ precursor (SOP) daughter cells in developing nota in the fly. They suggest that the altered

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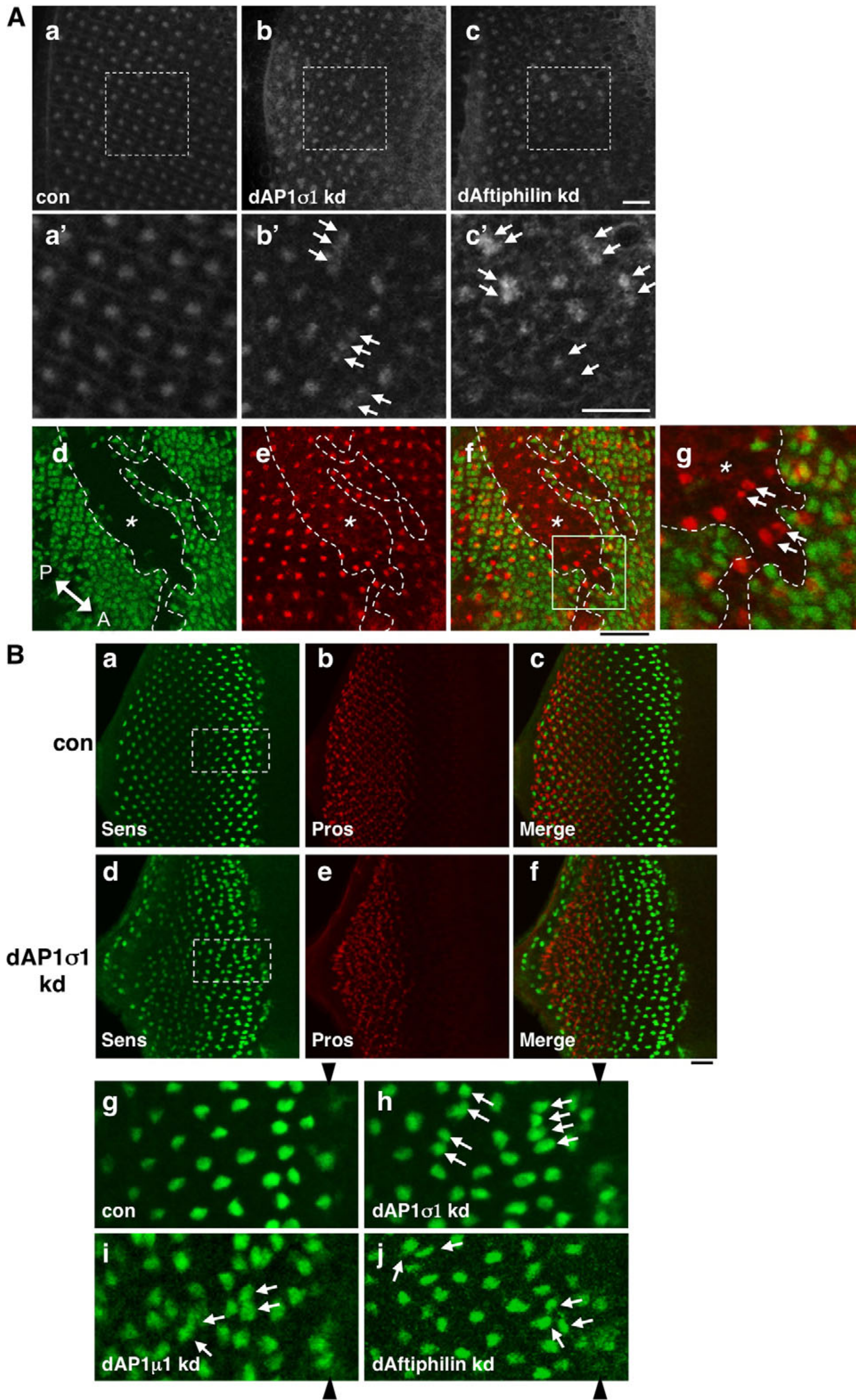

Fig. 5. Misalignment of photoreceptor cells in the AP-1 knockdown flies occurs in the larval eye disc. (**A**) Alignment of rhabdomere structures in the eye disc of control *ey*-GAL4/SM1 flies(con; a,a′), AP1σ1 knockdown flies (kd; b,b′) and Aftiphilin knockdown flies (c,c′). The photoreceptor cells of the eye antennal discs dissected from the 3rd instar larva were visualized with Alexa-Flour-594-conjugated phalloidin. Higher magnification images of the boxed areas in a–c are shown in a′–c′. (d–g) Alignment of rhabdomere structures is examined in clones for $AP47^{SHE11}$. Areas for the mutant clones (asterisks) are identified by the lack of GFP signal (green), while rhabdomeres are labeled by phalloidin signal (red). Boxed area in f is shown in g as a higher magnification image. Disordered and multiplied rhabdomeres are indicated by arrows. Posterior (P) and anterior (A) directions are indicated by the double-sided arrow. (**B**) Specification of the R8 photoreceptor neurons is affected in *Drosophila* AP-1 and Aftiphilin knockdown eye discs. The eye discs prepared from the 3rd instar larva of control (a,b,c) and AP1σ1 knockdown flies (d,e,f) were stained with an R8 marker (anti-Sens; green in a,c,d,f) and an R7 marker (anti-Pros; red in b,c,e,f). (g–j) The alignment of R8 photoreceptor neurons in AP-1 or Aftiphilin knockdown eye discs. The eye discs prepared from control (g) or from AP1σ1 (h), AP1μ1 (i) and Aftiphilin (j) knockdown flies were stained with anti-Sens antibody. Boxed areas in a,d are shown in g,h as enlarged images. Arrowheads indicate the MF regions. Scale bars: 10 μm.

trafficking of Notch is primarily due to increased recycling of the Notch regulator Spdo from the recycling endosomes to the plasma membrane, and that the mislocalization of Notch to the cell surface caused the gain-of-function phenotype in the AP-1 mutants (Benhra et al., 2011). By contrast, in our current study a clear loss-of-function phenotype of Notch was observed by depletion of AP-1 or Aftiphilin in the developing eyes.

This discrepancy is probably due to the different mechanisms by which intracellular trafficking of Notch is regulated in different tissues. Here, we focused on Scabrous as a candidate for a Notch regulator that is affected by AP-1 or Aftiphilin depletion. Scabrous is a glycosylated secretory protein expressed in the R8 neurons, and *sca* mutation as well as AP-1-depletion causes duplication of R8 and other photoreceptor neurons (Mlodzik et al.,

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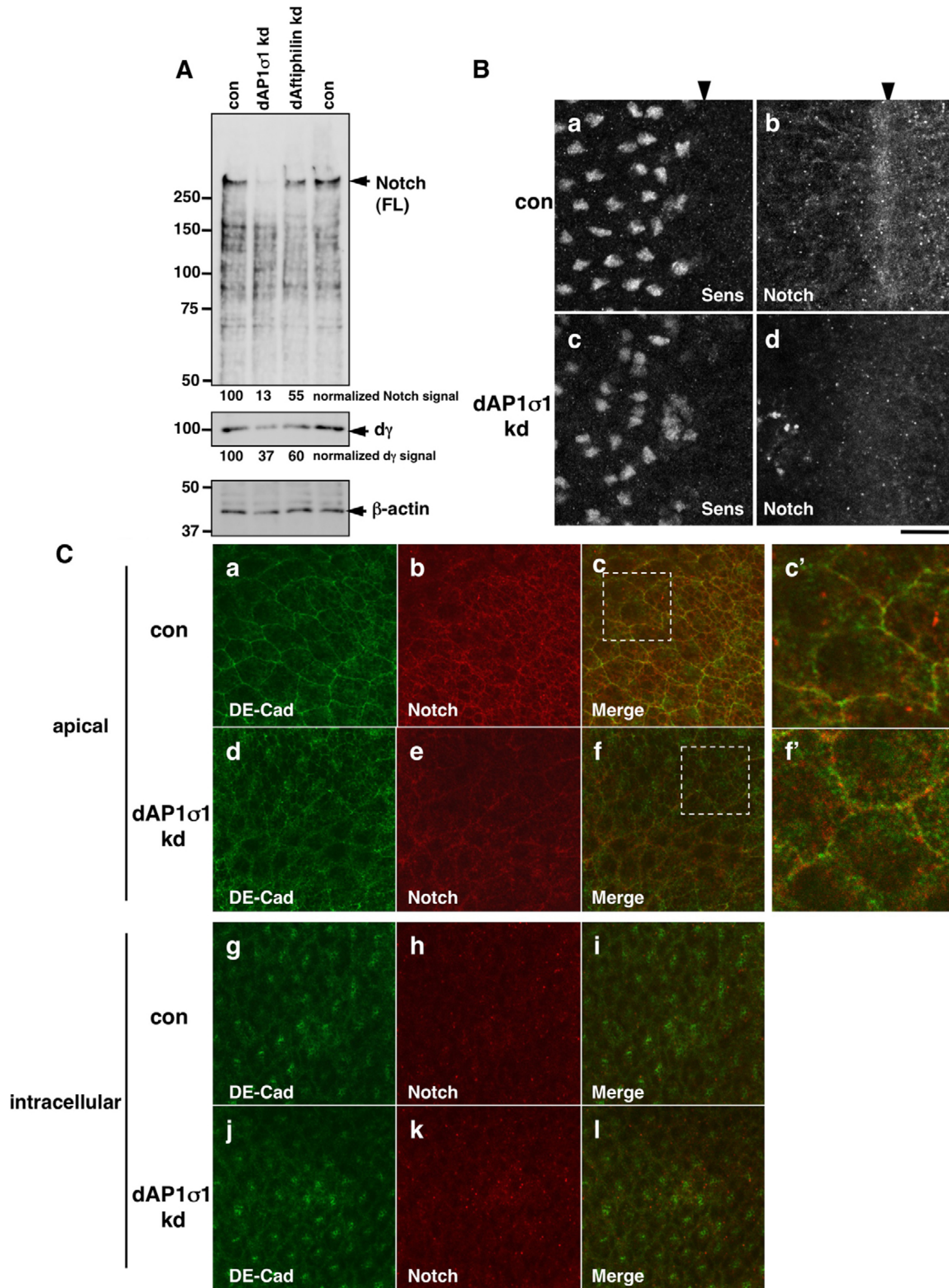

Fig. 6. Reduced expression of full-length Notch in the AP-1 or Aftiphilin knockdown eye discs. (**A**) Eye-discs dissected from the 3rd instar larva of control (con) or of AP1σ1 or Aftiphilin knockdown (kd) flies were subjected to immunoblotting using antibody against Notch, γ-adaptin (dγ) or β-actin. The relative intensity of the Notch and γ-adaptin signals normalized with that of β-actin is indicated. (**B**) Reduced expression of Notch in the eye disc of AP1σ1 knockdown larva. Indirect immunofluorescent microscopy of Notch and Sens in the eye discs prepared from control (a,b) or AP1σ1 knockdown (c,d) 3rd instar larva, were carried out. Projected images generated from confocal images are shown. Arrowheads indicate MFs. (**C**) Control (a–c,g–i) and AP1σ1 knockdown (d–f,j–l) eye discs were stained for DE-cadherin (green in a,c,d,f,g,i,j,l) and Notch (red in b,c,e,f,h,i,k,l). Confocal images were captured at the level of apical side (a–f) or the middle of cells (g–l). Boxed areas in c,f are shown in c′,f′, respectively, as higher magnification images. Scale bars: 10 μm.

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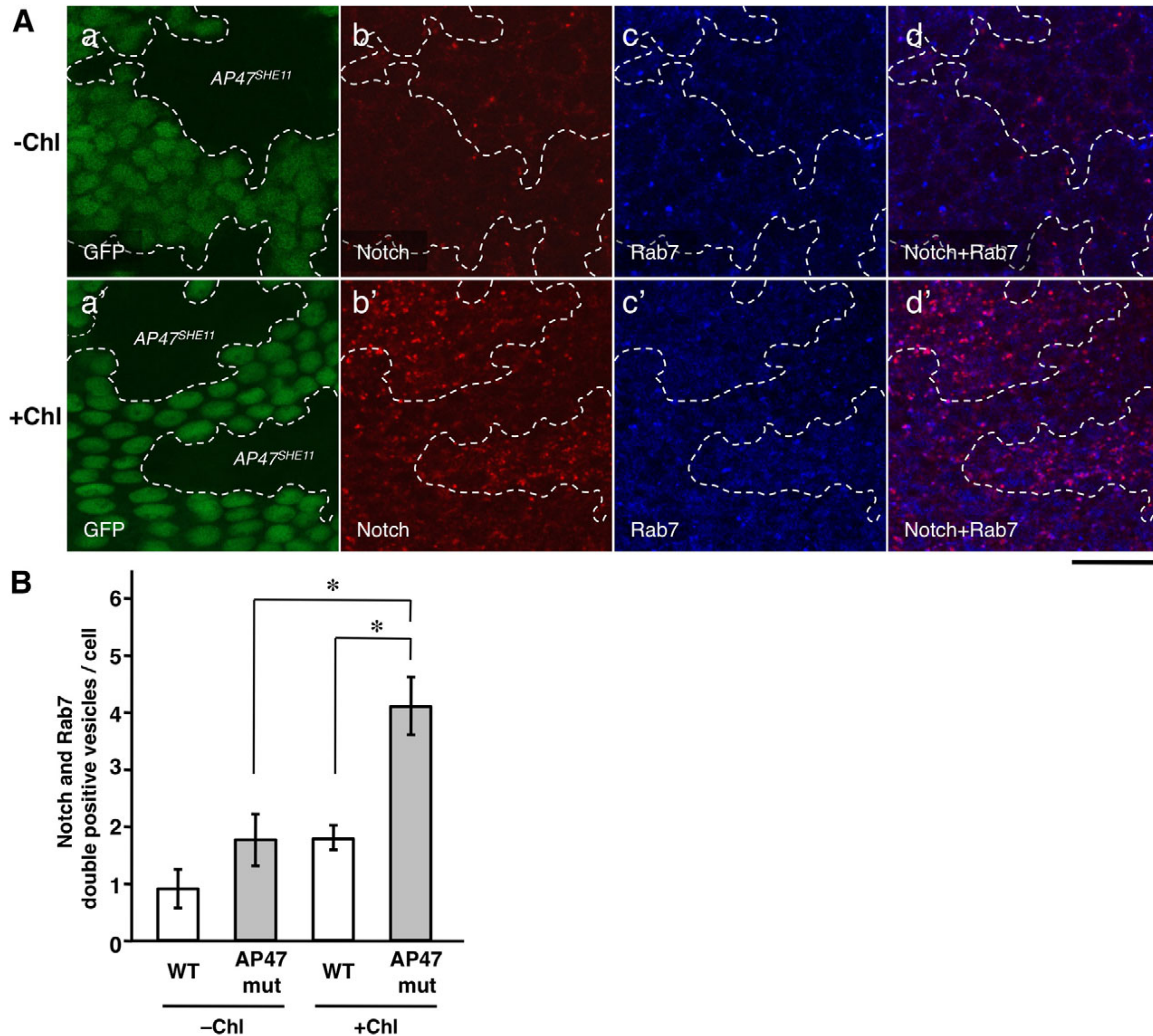

Fig. 7. Mistargeting of Notch to endosomal and lysosomal compartments in *Drosophila* AP-1 deficient cells. (**A**) $AP47^{SHE11}$ mosaic eye discs were cultured in the absence (a–d) or presence (a′–d′) of 200 µM chloroquine (Chl) for 10 hours as described in Materials and Methods, followed by immunostaining with antibodies against the Notch extracellular domain (C458.2H; red in b,d,b′,d′) and Rab7 (blue in c,d,c′,d′). Clones for $AP47^{SHE11}$ mutant are identified by the lack of GFP signal (a,a′). (**B**) The number of Notch/Rab7double-positive vesicles per wild-type (WT) or $AP47^{SHE11}$ mutant (AP47 mut) cell, with or without chloroquine treatment, was quantified in three independent samples. Values indicate mean ± s.d. $^{*}P<0.05$, determined by a two-tailed Student's *t*-test.

1990; Baker and Zitron, 1995; Lee et al., 1996) (supplementary material Fig. S8). In addition, Scabrous was also shown to bind to the extracellular domain of Notch and to stabilize Notch at the cell surface (Powell et al., 2001). More recently, Burgess and coworkers reported that *Drosophila* AP-1 functions together with clathrin in the biogenesis of mucin-containing secretory granules in the salivary gland (Burgess et al., 2011). Because Scabrous was shown to accumulate in the intracellular compartments in the AP-1-deficient eye discs (supplementary material Fig. S7), our observations in the current study suggest that a defect in the secretion of Scabrous and/or other regulatory proteins causes the instability of Notch at the cell surface, which leads to degradation of Notch in the endosomal and lysosomal compartments. The decrease in the amount of Notch on the cell surface then causes defects in the lateral inhibition mechanism required for the photoreceptor cell specification during eye development.

In addition to the tissue-specific regulation of Notch trafficking, Notch signaling could also be regulated in several ways in the intracellular trafficking pathways. In the AP-1-depleted eye antennal discs, Notch was accumulated at the late endosomal–lysosomal compartment upon treatment with the lysosomal inhibitor chloroquine, suggesting that Notch is missorted for its lysosomal degradation (Fig. 7 and supplementary material Fig. S5). Vaccari and Bilder recently showed that defects in endocytic trafficking caused by mutations of vps25, a component of the ESCRT-II complex, caused endosomal accumulation of Notch and enhanced Notch signaling (Vaccari and Bilder, 2005). This suggests that the cellular output of Notch signal could be affected drastically in several ways through alterations in the intracellular transport machineries for Notch protein. Finally, we cannot exclude the possibility that Notch is a cargo molecule for *Drosophila* AP-1,

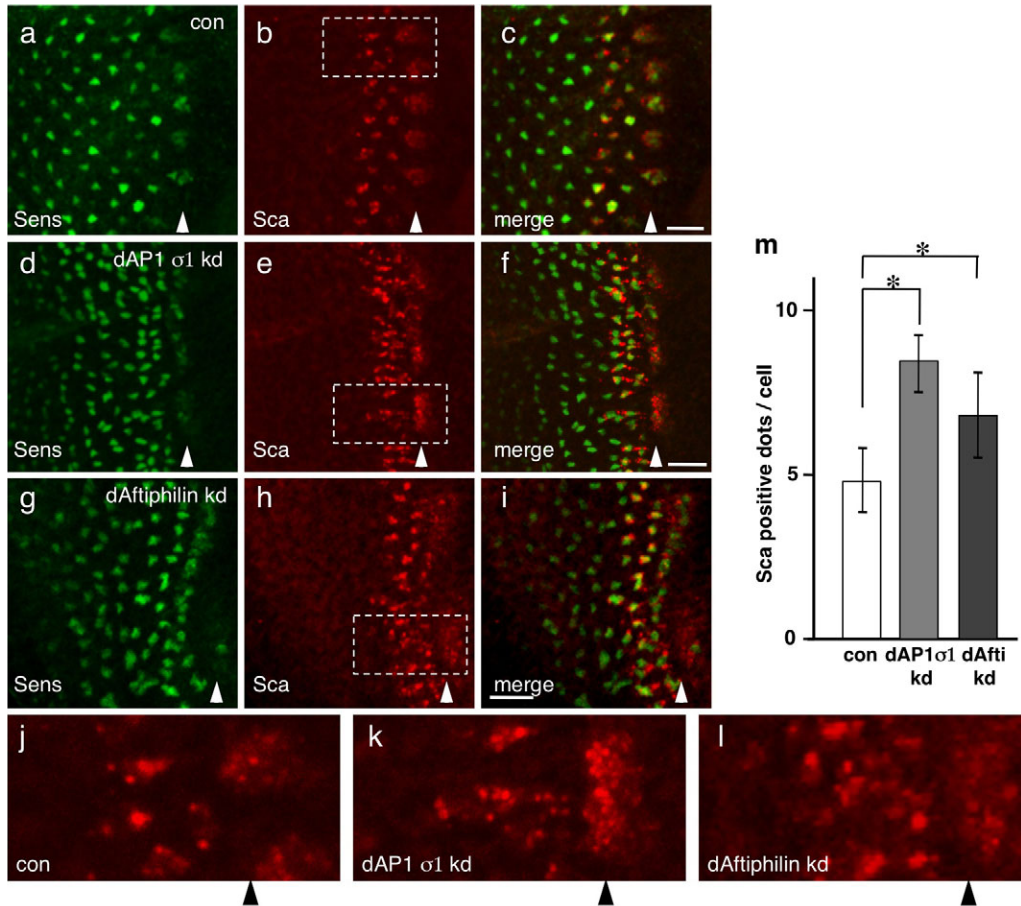

Fig. 8. Redistribution and accumulation of Scabrous in the AP-1 or Aftiphilin knockdown eye discs. Eye discs prepared from control (con) or from AP1σ1 or Aftiphilin knockdown (kd) larva were stained with anti-Sens (green in a,c,d,f,g,i) and anti-Scabrous (Sca) (red in b,c,e,f,h,i). Higher magnification images of the rectangular areas in b,e,h are shown in j,k,l, respectively. (m) The number of Sca-positive dots in each Sca-expressing cell near the MF was counted. Averaged numbers (mean ± s.d.) in three independent samples are shown. $^*P<0.05$, determined by a two-tailed Student's *t*-test. Arrowheads indicate the MF regions. Scale bars: 10 μm.

although no direct interaction between AP-1 and the cytoplasmic tail of Notch has been observed in our laboratory so far with biochemical approaches (unpublished observations).

In conclusion, *Drosophila* AP-1 plays a crucial role in Notch stability in vivo. We infer that *Drosophila* AP-1 is involved in the intracellular trafficking of tissue-specific regulators of Notch at the TGN or endosomal compartments, as proposed by Benhra and colleagues (Benhra et al., 2011). Notch trafficking can be regulated by several mechanisms, and a particular regulatory mode would predominate according to the context of the development. Further analysis on the precise molecular mechanisms by which *Drosophila* AP-1 and Aftiphilin are involved in the sorting of these signaling molecules will uncover the physiological functions of these adaptor proteins in vivo.

Materials and Methods

Cell culture and transfection

The Schneider S2 cell line (Schneider, 1972) was obtained from Invitrogen (Carlsbad, CA) and maintained in Schneider's medium supplemented with 10% fetal bovine serum (FBS), 50 U/ml penicillin and 50 μg/ml streptomycin. Transfection of S2 cells was performed with FuGene6 HD (Roche Molecular Biochemicals, Indianapolis, IN) or cellfectin (Invitrogen). For isolation of stable transfectants, S2 cells were co-transfected with 2 μg of expression constructs and 0.2 μg of pCoBlast (Invitrogen). At 24 hours after transfection, cells were transferred to fresh culture medium containing 10 μg/ml blasticidin (Invitrogen) and maintained for an additional 10–14 days to obtain stable transfectants.

Fly strains

The fly strain FRT82B, *AP47*^{SHE11}/TM6 *Tb Sb* was kindly provided by Roland Le Borgne, IGDR, Rennes, France (Benhra et al., 2011) and *y w ey*-FLP;;FRT82B *ubi*-GFP was a kind gift from Hiroshi Kanda (Keio University, Japan). The following *Drosophila* strains used in this study were distributed by National Institute of Genetics (Shizuoka, Japan): *actin*-GAL4, *GMR*-GAL4, *ey*-GAL4, *UAS*-*lacZ*, *UAS*-GFP, *UAS*-CG31072/LERP-RNAi, *UAS*-CG3002/dGGA-RNAi, *UAS*-CG9388/AP47 (AP1μ1)-RNAi, *UAS*-CG5864/AP1σ1-RNAi, *UAS*-CG9113/AP1γ1-RNAi, *UAS*-CG12532/BAP-RNAi, *UAS*-CG8358-RNAi, *UAS*-CG8385/Arf79F-RNAi, *UAS*-CG11027/Arf102F-RNAi and *UAS*-CG9012/CHC-RNAi. Other UAS-RNAi strains were purchased from the Vienna Drosophila RNAi Center (Vienna, Austria). Detailed information on the strains is shown in Table 1.

Antibodies and reagents

Mouse monoclonal antibodies to the V5 and HA (clone HA.11) epitope tags were purchased from Invitrogen and Covance (Princeton, NJ), respectively. Rabbit polyclonal antibodies against *Drosophila* Aftiphilin were generated with an antigenic peptide (NH_2-CRGLSNPPNQEESPHQWG-COOH) (MBL, Japan) and purified with affinity chromatography. Rabbit polyclonal antibody against *Drosophila* GGA was described previously (Kametaka et al., 2010). Rabbit antibodies against *Drosophila* γ-adaptin and GGA were kindly provided by Jennifer Hirst, CIMR, Cambridge (Hirst et al., 2009). Rat monoclonal antibody against p120 and rabbit polyclonal antibody against *Drosophila* GM130 were described previously (Yano et al., 2005). Guinea pig anti-Senseless antibody was kindly provided by Hugo Bellen, BCM, Houston, TX (Nolo et al., 2000). Mouse anti-Notch (C17.9C6 and C458.2H), anti-DE-cadherin (DCAD2), anti-Scabrous (sca1), anti-chaoptin (24B10), anti-Elav (9F8A9) and anti-Prospero (MR1A) monoclonal antibodies were provided by the Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA). Anti-Rab7 rabbit polyclonal antibody was provided by Akira Nakamura, RIKEN CDB, Kobe, Japan (Tanaka and Nakamura, 2008). Protease inhibitor cocktail was purchased from Roche, and chloroquine and 20-hydroxyecdysone were purchased from Sigma-Aldrich (St Louis, MO). Alexa-Fluor-594-conjugated phalloidin and Hoechst 33342 were purchased from Invitrogen.

Cloning of *Drosophila* genes and construction of plasmids

The ORF region of CG8538/Aftiphilin was amplified by PCR from the cDNA pool derived from S2 cells, and cloned into pAc5.1-A vector (Invitrogen) at *Kpn*I-*Xho*I sites to generate pAc-dAftiphilin-full-V5. Truncation mutants of *Drosophila*

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Aftiphilin (amino acid residues 151–1035, 301–1035, 451–1035, 601–1035 and 751–1035) and the ear domains of *Drosophila* γ-adaptin (amino acid residues 853–982) and GGA (amino acid residues 542–660) were cloned into the *Bam*HI-*Sal*I sites of pGEX6P-1 (GE healthcare) or pGAD-C1 vectors using standard techniques. Site-directed mutagenesis to generate point mutants within the *Drosophila* γ-ear was carried out using QuikChange XL site-directed mutagenesis system (Stratagene, La Jolla, CA) and these constructs were verified by DNA sequencing. pAc-dAP1μ1-V5 and pAc-dAP2μ2-V5 were described previously (Chaudhuri et al., 2007). pGAD-human γ1, mouse γ2, human GGA1-GAE, GGA2-GAE and GGA3-GAE were provided by Juan Bonifacino, NICHD/NIH, Bethesda, MD (Mattera et al., 2003).

Immunofluorescence and histology

Indirect immunofluorescent microscopy was essentially performed as described previously (Kametaka et al., 2005). Briefly, S2 cells were cultured on coverslips coated with poly-L-lysine, and fixed with 4% paraformaldehyde (PFA) in 0.12 M phosphate buffer (pH 7.2) at room temperature for 20 minutes. After permeabilization with 0.1% Triton X-100 for 5 minutes, blocking was carried out with PBS containing 1 mg/ml bovine serum albumin (BSA) at room temperature for 20 minutes. Then the cells were incubated with appropriate primary antibodies diluted in PBS for 1 hour, followed by treatment with secondary antibodies conjugated with fluorescent dye (Invitrogen).

For immunostaining of eye discs, the dissected eye antennal imaginal discs were fixed in 4% PFA in PBS for 20 minutes followed by incubation with PBS containing 0.05% Triton X-100 and 5% normal goat serum for 2 hours at 4℃. The tissues were then incubated with the primary antibodies for 18 hours at 4℃, washed four times with PBS containing 0.1% Triton X-100 and treated with the secondary antibody for 2 hours at room temperature. When required, nuclei were additionally labeled with 1 μg/ml Hoechst 33342. Fluorescent images were captured with an FV1000 confocal microscope (Olympus, Japan). For transmission electron microscopy, head parts of 1-day-old adult flies were dissected and fixed by immersing in the 0.1 M phosphate buffer (pH 7.4) containing 2% PFA and 2% glutaraldehyde overnight at 4℃. Following OsO_4 fixation, embedding and sectioning procedures were carried out as described previously (Waguri and Komatsu, 2009). Ultrathin sections were observed with a transmission electron microscope (JEM-1200EX, JEOL, Tokyo, Japan). For scanning electron microscopy, the head part was dissected, dried overnight and observed with a JSM-5800 scanning electron microscope (JEOL).

Pull-down assay

Bacterially expressed GST-fusion proteins were purified and used for pull-down experiments as previously described (Kametaka et al., 2007). Briefly, 25 μg of purified GST, GST–γ-ear and GST–GGA-GAE fusion proteins were bound to glutathione Sepharose CL-4B (GE healthcare, Piscataway, NJ) to prepare the GST beads. The cell lysate prepared from the S2 cells transiently expressing *Drosophila* Aftiphilin-V5 was incubated with the GST beads for 18 hours at 4℃ with gentle rotation. After the incubation, beads were washed with PBS containing 0.25% Triton X-100 three times and the bound protein subjected to immunoblotting.

Yeast two-hybrid assay

Yeast two-hybrid assays were performed essentially as described previously (Kametaka et al., 2010). Briefly, yeast AH109 cells (Clontech, Mountain View, CA) were maintained and transformed with appropriate combination of the bait and the prey vectors with the standard protocols. Growth of the transformants on SD-WL (lacking Trp and Leu) and SD-WLHA (lacking Trp, Leu, His and adenine) plates was assayed 3–5 days after spotting of the cells.

Immunoprecipitation

Co-immunoprecipitation analysis was carried out as described previously (Mardones et al., 2007). In brief, cells were lysed in ice-cold lysis buffer [0.5% Triton X-100, 1 × PBS and 1 × Protease inhibitor cocktail (Roche)] and rotated with Protein-A–Sepharose (GE healthcare) for 30 minutes at 4℃. After centrifugation at 15,000 ***g*** for 15 minutes, the clear supernatant was recovered as a total lysate. The total lysate was incubated with the appropriate antibody for 18 hours followed by incubation with Protein-A–Sepharose for 2 hours at 4℃. The immunocomplex captured by Protein-A–Sepharose was precipitated by centrifugation and the beads washed with ice-cold lysis buffer four times, then the bound protein was eluted by boiling the beads in 1 × Laemmli sample buffer for 5 minutes.

Organ culture of imaginal discs

Culture of the eye discs was carried out as described previously (Vaccari and Bilder, 2005; Ting et al., 2007). In brief, eye antennal discs dissected from the third instar larvae were cultured in Shields and Sang M3 insect medium (Sigma) supplemented with 1% FBS and 2 μg/ml 20-hydroxyecdysone (Sigma), in the presence or absence of 200 μM chloroquine.

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