Fab1 Phosphatidylinositol 3-Phosphate 5-Kinase Controls Trafficking but Not Silencing of Endocytosed Receptors

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The trafficking of endocytosed receptors through phosphatidylinositol 3-phosphate [PtdIns(3)P]-containing endosomes is thought to attenuate their signaling. Here, we show that the PtdIns(3)P 5-kinase Fab1/PIKfyve controls trafficking but not silencing of endocytosed receptors. *Drosophila fab1* mutants contain undetectable phosphatidylinositol 3,5-bisphosphate levels, show profound increases in cell and organ size, and die at the pupal stage. Mutant larvae contain highly enlarged multivesicular bodies and late endosomes that are inefficiently acidified. Clones of *fab1* mutant cells accumulate Wingless and Notch, similarly to cells lacking Hrs, Vps25, and Tsg101, components of the endosomal sorting machinery for ubiquitinated membrane proteins. However, whereas *hrs*, *vps25*, and *tsg101* mutant cell clones accumulate ubiquitinated cargo, this is not the case with *fab1* mutants. Even though endocytic receptor trafficking is impaired in *fab1* mutants, Notch, Wingless, and Dpp signaling is unaffected. We conclude that Fab1, despite its importance for endosomal functions, is not required for receptor silencing. This is consistent with the possibility that Fab1 functions at a late stage in endocytic receptor trafficking, at a point when signal termination has occurred.

INTRODUCTION

Cell growth, survival, proliferation, and differentiation are controlled by signals that activate their cognate receptors on the cell surface. Important examples include the soluble ligand Wnt (and its *Drosophila* homologue Wingless) and the membrane bound ligand Delta, which bind to G protein-coupled receptors and Notch receptors, respectively, on receiving cells. During development and normal physiology, the levels of the ligands and their receptors are tightly controlled in time and space (Sorkin and Von Zastrow, 2002; Gonzalez-Gaitan, 2003; Piddini and Vincent, 2003; Polo *et al.*, 2004).

Receptor density at the cell surface is an important determinant of signaling responses, and there are both slow and fast mechanisms attenuating receptor levels. Transcriptional down-regulation is a slow and long-lasting mechanism, whereas posttranslational modification and/or internalization represent fast ways to reduce the amounts of functional receptors on the cell surface. Internalization of many receptors, including Notch and Wnt receptors, is followed by their

transport from endosomes to lysosomes, where they become degraded, resulting in a transient reduction in the ability of cells to receive signals. Adding to the complexity of signaling regulation is the fact that ligand-bound receptors may also signal from endosomal membranes, and their signaling output from endosomes may differ from the output triggered from the plasma membrane (Ceresa and Schmid, 2000; Sorkin and Von Zastrow, 2002).

The key roles of the endocytic pathway in cell signaling are highlighted by the analyses of mutants interfering with endocytic trafficking. Such an example is provided by Hrs, a protein that sorts ubiquitinated receptors into intraluminal vesicles of multivesicular bodies (MVBs), destined for degradation in lysosomes. *Drosophila hrs* mutants show impaired sorting of receptors into MVBs, causing their accumulation in early endosomes (Lloyd *et al.*, 2002; Jekely and Rorth, 2003). In *hrs* mutants, Dpp (a transforming growth factor- β homologue) and epidermal growth factor receptor signaling is enhanced, presumably because the activated receptors have a prolonged residence time in the limiting membrane of endosomes. Likewise, mutations of two subunits of the endosomal sorting complex required for transport (ESCRT)-I and -II, Tsg101 and Vps25, which are thought to function immediately downstream of Hrs, cause endosomal accumulation of receptors and tumor-like overproliferation in a cell nonautonomous manner due to increased Notch signaling (Moberg *et al.*, 2005; Thompson *et al.*, 2005; Vaccari and Bilder, 2005). This supports the view that proper endocytic traffic has an important antitumorigenic function (Bache *et al.*, 2004; Polo *et al.*, 2004).

Hrs is recruited to endosome membranes by binding the phosphoinositide (PI) phosphatidylinositol (PtdIns) 3-phosphate [PtdIns(3)P], formed by phosphorylation of PtdIns by

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Abbreviations used: BSA, bovine serum albumin; EM, electron microscopy; ESCRT, endosomal sorting complex required for transport; GFP, green fluorescent protein; HRP, horseradish peroxidase; MVB, multivesicular body; OGD, Oregon green dextran; PI, phosphoinositide; TRD, Texas Red dextran.

a class III PI 3-kinase (Raiborg *et al.*, 2001). PtdIns(3)P is specifically localized to endosomal membranes (Gillooly *et al.*, 2000) and not only recruits Hrs but also several other proteins containing FYVE or PX domains (Ellson *et al.*, 2002; Stenmark *et al.*, 2002). Class III PI 3-kinase and PtdIns(3)P are thus crucial regulators of endocytic trafficking, mediating endosome fusion as well as degradative sorting, recycling, and retrograde trafficking to the biosynthetic pathway (Lindom and Stenmark, 2006). PtdIns(3)P is metabolized by dephosphorylation and by lysosomal lipases (Stenmark and Gillooly, 2001). In addition, this PI can be phosphorylated in the 5-position of the inositol headgroup, giving rise to phosphatidylinositol 3,5-bisphosphate [PtdIns(3,5)P₂] (Dove *et al.*, 1997; Cooke, 2002; Efe *et al.*, 2005). The kinase catalyzing this phosphorylation, Fab1, was first characterized in yeast. *Saccharomyces cerevisiae* *fab1* mutants have abnormally enlarged vacuoles and show impaired trafficking of the ubiquitinated cargo carboxypeptidase S to the vacuole lumen (Odorizzi *et al.*, 1998). Fab1 is evolutionarily conserved, and overexpression of a kinase-dead mutant of the mammalian Fab1 homologue PIKfyve in cultured cells has been reported to inhibit fluid-phase transport of endocytic markers but not recycling/degradation of endocytosed receptors or sorting of procathepsin D (Ikonov *et al.*, 2001, 2003). Moreover, PIKfyve has been found to be phosphorylated by the PI 3-kinase-regulated protein kinase, PKB, after insulin stimulation, and PIKfyve colocalizes with a highly motile subpopulation of vesicles containing insulin-responsive aminopeptidase (Berwick *et al.*, 2004).

These findings indicate that Fab1/PIKfyve plays a role in controlling specific membrane trafficking processes, but its functions in signal termination and in the physiology of a multicellular organism are not known. To address this, we have generated *Drosophila fab1* mutants and studied their phenotype with respect to survival, growth, membrane trafficking and cell signaling. We find that the activity of *Drosophila* Fab1 is essential for development and cell volume control and that its inactivation leads to endosomal accumulation of Wingless and Notch. Remarkably, this accumulation is not accompanied by increased signaling, indicating that Fab1, unlike Hrs and ESCRT-I and -II, is not involved in receptor silencing.

MATERIALS AND METHODS

Molecular Biology and Generation of Transgenic Lines

The pUAS-*fab1* P-element vector was generated by cloning the *fab1* cDNA (GH01668; Research Genetics, Huntsville, AL) into pUASp by using AscI/XbaI (Rorth *et al.*, 1998). The P-element vector pCaSpeR4 carrying a ubiquitously expressed *tubulin* promoter was modified by addition of enhanced green fluorescent protein (EGFP) from pEGFP-C2 (Clontech, Mountain View, CA) into the NotI and XbaI sites (Basler and Struhl, 1994). Atg18 was amplified by PCR from pGEX-4T1-Atg18 (Dove *et al.*, 2004) by using specific primers encoding 5' and 3' XbaI and StuI sites, respectively, and ligated into the XbaI/StuI-digested pCaSpeR4-EGFP to generate pCaSpeR4-EGFP-Atg18 (here called tGFP-Atg18). The plasmids were injected into *w¹¹¹⁸* embryos for transformation. A minimum of four independent lines were established and tested in each case. The mutations in the *fab1¹⁸*, *fab1²¹*, and *fab1³¹* alleles were identified by sequencing at least two overlapping PCR products amplified from homozygous mutant genomic DNA in each case. Primer sequences are available upon request. For antibody production, a construct comprising the N-terminal 400 residues of Fab1 as a C-terminal fusion with maltose binding protein (MBP) was prepared in pMAL-C2 (New England Biolabs, Beverly, MA).

Drosophila Stocks and Genetics

Drosophila stocks included UAS-GFP-Rab5, UAS-GFP-Rab7, UAS-myc-GFP-2xFYVE (Entchev *et al.*, 2000), *Df(2R)^{w30}* (Mohr and Gelbart, 2002), *nub-Gal4* (Calleja *et al.*, 1996), *hrs²⁸*, UAS-hrp-Lamp1 (Lloyd *et al.*, 2002), and UAS-hrp-wg (Dubois *et al.*, 2001). For description of other alleles and balancer chromosomes, see FlyBase at <http://flybase.bio.indiana.edu/>. Crosses were performed at 25°C (unless otherwise specified), and imaginal disk and ovar-

ian clones were generated by heat shocking first- (L1) and second-stage (L2) larvae for 1 h at 37°C when hs-flip was used as a source of Flip recombinase.

Antibody Generation

Polyclonal antiserum against *Drosophila* Lamp1 (CG3305) was generated by injection of rabbits with a synthetic peptide of 22 amino acids corresponding to the C-terminal cytoplasmic tail SYLCARRRSTRSGYMSF. Polyclonal antiserum toward the N-terminal 400 amino acids of Fab1 was generated by injection of a purified bacterially expressed MBP-Fab1 fusion protein into rabbits.

Immunohistochemistry, Dextran Uptake, and Microscopy

Dextran and bovine serum albumin (BSA) uptake experiments in imaginal disks were performed as described previously (Entchev *et al.*, 2000). Imaginal disks and ovaries were fixed and stained by standard procedures using the following antibodies: mouse anti-Notch (1:40), DI (1:20), Cut (1:50), Hindsight (1:40), Disk Large (1:40), and Wg (1:1000) (all from the Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA), rabbit anti-*Drosophila* Lamp1 (1:1000), rabbit anti-horseradish peroxidase (HRP) (1:400) (Sigma-Aldrich, Steinheim, Germany), affinity-purified rabbit anti-Fab1 (1:250), rat anti-Spalt (Sal; 1:500) (De Celis and Barrio, 2000), guinea pig anti-Senseless (Sens; 1:1000) (Nolo *et al.*, 2000), rabbit anti-phosphorylated Mothers Against Dpp (Mad; 1:1000) (Tanimoto *et al.*, 2000), guinea pig anti-Hrs (Hrs; 1:1000) (Lloyd *et al.*, 2002), guinea pig anti-Eyegone (Ey; 1:1000) (Aldaz *et al.*, 2003), rat anti-Distalless (Dll; 1:500) (Vachon *et al.*, 1992), and mouse anti-poly- and monoubiquitin (Affiniti Research Products, Exeter, United Kingdom). Cy2-, Cy3-, and Cy5-conjugated secondary antibodies (1:1000) were from Jackson ImmunoResearch Laboratories (West Grove, PA). Phalloidin (1:50) was used to detect actin, TOTO-3 (1:100) to stain DNA, and LysoTracker Red DND-99 (1:200) to detect acidic compartments (all from Invitrogen, Carlsbad, CA). Confocal images were recorded using a Zeiss LSM510 Meta microscope, and images of adult structures were obtained on a Leica MZ FLIII Leica microscope equipped with a Leica DC480 camera (Leica, Wetzlar, Germany), and processed using Adobe Photoshop, version 7.0 (Adobe Systems, Mountain View, CA). The area of ommatidia from eight eyes (4 animals) of each genotype was measured from images obtained at identical conditions using Adobe Photoshop, version 7.0.

Electron Microscopy

Garland cells and wing disks were prepared for conventional plastic embedding according to the following protocol. Cells and tissue were fixed in 2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, for 1 h at room temperature. Postfixation was performed in 1% OsO₄ and 1.5% KFeCN (1 h), followed by en bloc staining in 4% uranyl acetate (30 min), dehydration in graded alcohol concentrations, and embedding with Epon 812. Ultrathin sections were cut on a Leica Ultracut, counterstained with 1% lead citrate and observed in a Philips CM10 at 60–80 kV. Substrate reactions in eye disks expressing HRP-Lamp or HRP-wg were done as follows. After initial glutaraldehyde fixation, the disks were washed in phosphate buffer, incubated for 5 min with 5 mg/ml diaminobenzidine (DAB), followed by 45 min incubation with DAB added to H₂O₂ to a final concentration of 0.02%. After washing, the samples were further processed for postfixation as described above. Wing disks were also prepared for cryosectioning after fixation in 4% formaldehyde and 0.1% glutaraldehyde in 0.1 M phosphate buffer. The eye disks were embedded in 10% gelatin followed by infusion with 2.3 M sucrose (1 h), mounted on silver pins, and frozen in liquid nitrogen. Ultrathin sections were obtained at −110°C, picked up with a 1:1 mixture of 2.3 M sucrose and 2% methyl cellulose. Sections were then embedded with 2% methyl cellulose and 0.4% uranyl acetate and observed in the microscope as described above.

RESULTS

Drosophila Contains a Functional Fab1 Homologue That Localizes to Endosomes and Is Necessary for Viability and Cell Size Control

A single *fab1* homologue (CG6355, here named *fab1*) was identified in the *Drosophila melanogaster* genome. Sequence comparisons between a 6-kilobase (kb) cDNA clone, GH01668, and the genomic sequence revealed a gene structure of nine exons as predicted by the FlyBase consortium (Figure 1A). The longest open reading frame encodes a protein of 1809 amino acid residues with a conserved N-terminal FYVE domain and a C-terminal PtdIns(3)P 5-kinase (5K) domain.

Four ethyl methanesulfonate-induced alleles of a single complementation group were identified in the *fab1* region at cytological location 54F, uncovered by a small deficiency, *Df(2R)^{w30}* (Englund *et al.*, 1999). Genomic sequencing of three of the alleles *l(2)SJB⁸*, *l(2)SJB²¹*, and *l(2)SJB³¹* revealed

Figure 1. *Drosophila fab1* structure, alleles, phenotypes, and failure of recruitment of GFP-Atg18 to endocytic structures in *fab1* mutants. (A) Cartoons representing the *Drosophila fab1* cDNA (exons are in black and introns in white) and predicted protein structure including the

nonsense mutations in the *fab1* coding region, resulting in predicted truncated proteins lacking a functional kinase domain (Figure 1A). Heterozygotes of either of the three alleles over either *Df(2R)^{w30}* or *Df(2R)^{Pcl^{7b}}*, which both delete the *fab1* locus, died as late pupae or rare pharate adults in the process of hatching (Figure 1, A and B). Moreover, the lethal phase of *l(2)SJB⁸/l(2)SJB²¹*, *l(2)SJB⁸/l(2)SJB³¹*, or *l(2)SJB³¹/l(2)SJB²¹* was identical to that of *l(2)SJB⁸*, *l(2)SJB²¹*, or *l(2)SJB³¹* over *Df(2R)^{w30}*, suggesting they all represent null, or close to null alleles (Figure 1A). We hereafter refer to the *fab1* alleles as *fab1⁸*, *fab1²¹*, and *fab1³¹*, respectively.

We noticed that *fab1* pupae were larger than control animals, even though *fab1* larvae did not display an extended larval phase (Figure 1B). This size difference was also evident when comparing the sizes of the appendages of *fab1* homozygotes dissected from the pupal cases to those of wild-type pupae. The legs were of correct length but considerably thicker than in control animals. Loss of *fab1* can autonomously cause tissue-specific overgrowth, because mosaic adult flies (Newsome *et al.*, 2000) with heads consisting primarily of *fab1* mutant cells were viable and showed a clear enlargement of the head and eyes (Figure 1, C and D). The overgrowth phenotypes were also distinct in the larval tissues of *fab1* mutants. Salivary glands, gut tissue, brain

lobes, and imaginal disks looked larger and swollen compared with wild type (our unpublished data).

To address whether Fab1 is the responsible kinase for producing PtdIns(3,5)P₂ on endosomal structures, we took advantage of the fact that the yeast green fluorescent protein (GFP)-Atg18/Svp1 fusion protein can serve as an *in vivo* probe for the detection of PtdIns(3,5)P₂ lipids on endosomes (Dove *et al.*, 2004). In transgenic flies expressing GFP-Atg18 under the control of the ubiquitously expressed tubulin promoter, punctate structures were preferentially observed in the apical cytoplasm of eye imaginal disk cells (Figure 1, E–G). A partial colocalization was observed with LysoTracker (Figure 1E), which labels late acidic endosomes and lysosomes, and with endosomes labeled with internalized Texas Red dextran (TRD) (Figure 1, F and G), demonstrating that GFP-Atg18 can be found on late acidic endosomal structures. Like previously reported in *fab1* yeast mutants, the punctate localization of GFP-Atg18 was lost in *fab1* mutant cells and instead occurred diffusely in the cytoplasm (Figure 1H). Thus, Fab1 seems to be the sole kinase producing PtdIns(3,5)P₂ on endosomal membranes (Figure 1I).

To detect endogenous Fab1, we generated polyclonal antibodies against the N-terminal part of recombinant Fab1. Distinct punctate structures were labeled in Garland cells isolated from wandering L3 control animals, whereas no staining was observed in *Df(2R)^{w30}/Df(2R)^{Pcl^{7b}}* larvae, demonstrating the specificity of the antibody (Figure 2, A and B). We used the antibody to determine the subcellular localization of Fab1 relatively to markers of the endocytic machinery in wing imaginal disks and in Garland cells. Fab1 showed little colocalization with the fluid-phase marker Oregon green dextran (OGD) after a 5-min internalization in Garland cells (Figure 2C), whereas there was significant colocalization after a 40-min chase (Figure 2D). Moreover, Fab1 colocalized partially with markers of early (GFP-Rab5; Figure 2E) and late endosomes (GFP-Rab7; Figure 2H). Interestingly, only limited colocalization, but still proximity, was observed between Fab1 and Hrs (Figure 1F) and a probe specific for PtdIns(3)P (GFP-2xFYVE; Figure 1G), perhaps reflecting the rapid turnover of PtdIns(3)P on a subset of endosomal membranes (Gillooly *et al.*, 2000; Lloyd *et al.*, 2002; Wucherpfennig *et al.*, 2003). This finding is in agreement with the distribution of Fab1 in mammalian cells, where Fab1, Hrs, and PtdIns(3)P are found on different microdomains of endosomal membranes (Cabezas *et al.*, 2006). Close examination of unfixed *fab1* mutant Garland cells showed that these had up to 3 times their normal diameter (Figure 2I). Investigation of the imaginal wing disk epithelium from *fab1* mutant larvae revealed that the overall cell polarity was intact, whereas the cells were larger and showed a disorganized distribution of nuclei relative to wild-type wing disks (Figure 2, J and K). Together with the increased organ size in *fab1* mutants (Figure 1, B–D), this indicates that Fab1 exerts a negative cell size control but is not required for epithelial polarization.

Fab1 Is Necessary for Endosome Acidification and Efficient Fluid-Phase Transport to the Lysosome

Similarly to what has been reported for yeast and mammalian *fab1* mutant cells (Odorizzi *et al.*, 1998; Ikonov *et al.*, 2001), we found a strongly reduced lysosomal acidity in *fab1* mutant Garland cells (Figure 2I). To investigate the role of Fab1 in fluid-phase transport, trafficking of fluorescently labeled internalized dextran was investigated in wandering third instar Garland cells. After 5 min of uptake in control cells, OGD was observed in distinct punctate endocytic

Figure 1 (cont). N-terminal FYVE domain, chaperonin-like domain, and C-terminal kinase domain. The vertical bars with associated numbers indicate the position of the point mutations leading to premature stop codons in the respective alleles, *fab1⁸* (Q1308Stop), *fab1³¹* (L1321Stop), and *fab1²¹* (Q1521Stop). The complementation table shows *fab1* alleles over a small, *Df(2R)^{w30}*, and larger deficiency, *Df(2R)^{Pcl^{7b}}*, which both uncover the *fab1* locus. The lethal phase of the respective transheterozygotic combinations is indicated. pa, pharate adult; wL3, wandering L3 larva; nd, not determined. (B) *fab1* mutant pharate adults and dissected legs are larger than in control animals of a comparable stage of development (ventral and dorsal views of pupae are shown with arrows indicating body length). (C and D) Heads and eyes display tissue overgrowth in animals whose head consists mainly of *fab1* mutant cells (mutant cells in the eye lack red pigmentation; see *Materials and Methods*). The average ommatidium size was 1.25 times larger in *fab1* mutant eyes (*n* = 828 ommatidia, 8 eyes) than in control eyes (*n* = 952, 8 eyes). (E–E') Confocal image in which recruitment of GFP-Atg18 can be seen to punctate structures (arrowheads) and late endosomes and lysosomes (arrows) labeled with LysoTracker in a unfixed eye disk from an L3 larva. (F–F') Confocal image of apical endosomes labeled with a 20-min uptake of TRD in a fixed eye imaginal disk. Note that structures can be seen with TRD alone (asterisks, probably representing early endosomes) or together with GFP-Atg18 (arrows). Some structures label with GFP-Atg18 alone (arrowhead). (G–G' and H–H') Overview confocal images in which internalized dextran can be observed in apical developing photoreceptor cell clusters in both control and *fab1* mutant eye disks. Although GFP-Atg18 can be clearly seen concentrated on subcellular structures in control disks, no such subcellular accumulation can be seen in the mutant tissue; 12.6 ± 3.6% of GFP-Atg18 structures also labeled with TRD in control animals (*n* = 583 endosomes 5 eye imaginal disks), whereas no GFP-Atg18 structures (0%) were observed with *fab1* mutant discs (*n* = 303 endosomes, 5 eye imaginal disks). (I) Cartoon showing the conversion of PtdIns into PtdIns(3)P by PI 3-kinase (PI3K) and the subsequent conversion into PtdIns(3,5)P₂ by Fab1. The FYVE domain and the yeast protein Atg18 can specifically bind PtdIns(3)P or PtdIns(3,5)P₂, respectively, and serve as probes for detecting these lipids *in vivo*. Bars, 1 μm (C–E) and 2 μm (F–H). Genotypes are *fab1³¹/Df(2R)^{w30}* and *+/Df(2R)^{w30}* (B), *yw, ey-flp, GMR-LacZ/Y; FRT42D, p [mini-w]*, *l(2)clR11/FRT42D, fab1²¹, yw, ey-flp, GMR-LacZ/Y; FRT42D, p [mini-w]*, *l(2)clR11/+* (mutant and control heads in C and D), *w¹¹¹⁸, tGFP-Atg18/+* (E–G), and *fab1³¹/Df(2R)^{w30}; tGFP-Atg18/+* (H).

Figure 2. Fab1 subcellular localization and effect on lysosomal acidity. (A and B) Punctate subcellular staining of Fab1 was observed in isolated Garland cells from wandering L3 control animals but not from *Df(2R)^{w30}/Df(2R)Pcl^{7b}* mutants that lack the *fab1* genomic region. The dashed line indicates the cell membrane. (C and D) OGD was internalized in Garland cells for 5 min, and immunolocalization with Fab1 was scored after 0 (C) and 40 min (D). Fab1 overlaps extensively with OGD after 40 min, when OGD primarily labels late endosomes/lysosomes. (E–H) In apical confocal sections of epithelial cells of wing imaginal disks, extensive overlap is also observed with GFP-Rab5 on early and GFP-Rab7 on late endosomes, but Fab1 seems to be in proximity rather than in the same region as GFP-2xFYVE and Hrs on sorting endosomes/late endosomes. (I) Intensity of LysoTracker emission is a measure of acidity of intracellular late endosomes and lysosomes. Pictures are shown taken with the same confocal scanning settings of Garland cells from mutant animals in which the average acidity is dramatically decreased compared with the control. (J and K) In optical Z-sections of the epithelium from wing imaginal disks, more space and irregular organization between TOTO-3-labeled nuclei can be observed in mutant tissue compared with the control. The organization of apical adherence junctions where actin accumulates and the slightly more basally localized septate junctions containing Disk large (Dlg) seem normal. Bars, 5 μ m (A and B), 2 μ m (C and D), 1 μ m (E–H), 50 μ m (I), and 5 μ m (J and K). Genotypes are *w¹¹¹⁸* (A, C, D, F, I, and J), *w¹¹¹⁸/+; ptc-Gal4/UAS-GFP-Rab5* (E), *w¹¹¹⁸/+; ptc-Gal4/UAS-myc-GFP-dbFYVE* (G), *w¹¹¹⁸/+; ptc-Gal4/UAS-GFP-Rab7* (H), and *fab1³¹/Df(2R)^{w30}* (I and K).

structures close to the plasma membrane (Figures 2C and 3A). The predominant portion of OGD reached the more centrally located lysosomal compartment labeled with the pH-sensitive vital dye LysoTracker, after 40 min of chase (Figure 3B). OGD remained mainly in the periphery of the cells and failed to be transported to the weakly acidic compartments in *fab1* mutant cells, suggesting a failure of fluid-phase transport to the lysosomes (Figure 3, C and D). This is consistent with the finding that overexpression of a kinase-dead PIKfyve mutant inhibits lysosomal transport of endocytosed HRP in human embryonic kidney cells (Ikononov *et al.*, 2003).

Investigation of fluid phase uptake of gold-labeled BSA, which allows visualization of the endocytic pathway by electron microscopy (EM), revealed the presence of endocytosed tracer in lysosomes after 40-min uptake in control cells (Figure 3, E–G). In *fab1* mutant cells (Figure 3, H and I), the

gold particles accumulated in greatly enlarged endosomes (consistent with the fluorescence microscopy), whereas most lysosomes were devoid of the gold tracer. We conclude that, similar to the situation in yeast and mammalian cells, Fab1 is necessary for lysosomal acidification in *Drosophila*. This failure of acidification also applies for Rab7-positive MVBs (our unpublished data). Interestingly, a similar reduction of acidity, and increase in cell size has been observed in *hrs* mutant Garland cells (Lloyd *et al.*, 2002), but the transport of fluid phase tracer to lysosomes is intact in such cells (our unpublished data). The failure of transport of endocytic fluid phase tracer into the electron-dense structures observed by EM and LysoTracker-positive structures observed by immunofluorescence suggests a failure of endocytosed material to reach the lysosome or a failure of maturation of the endosome into a functional degradative compartment.

Figure 3. Altered fluid-phase transport and accumulation of cargo in a prelysosomal compartment in *fab1* mutants. (A and B) OGD accumulated in small peripheral vesicles after 5 min of uptake and reached late endosomal/lysosomal compartment after a 40-min chase period (arrows). (C and D) In *fab1* mutants, in contrast, OGD failed to reach acidified compartments after chase (note that detector gain of the red channel was increased to allow visualization of the more weakly acidified compartments in *fab1*³¹/Df(2R)^{w30} cells). (E–J) Electron micrographs of control (E–G) and *fab1* (H–J) Garland cells. (E and H) Low magnification overview shows normal-sized endosomes in control cells and greatly enlarged endosomes in the *fab1* cells (arrows in both micrographs). To further characterize the endocytic pathway, we incubated cells with BSA-Gold (arrowheads in all micrographs) for 40-min continuous incubation. (F and G) In control cells, we observed BSA-gold (arrowheads in enlarged inset of F and G) both in endosomal structures and lysosomal compartments. (I) In *fab1* mutants, BSA-gold localized to dramatically enlarged endosomes and was not observed in dense lysosomal structures. Note that only part of the endosome is depicted. (J) Enlarged view of boxed area in I shows gold particles associated with dense matter in endosome. n, nucleus; e, endosome; and l, lysosome. Bars, 2 μ m (A–D), 1 μ m (E and H), 100 nm (F), and 200 nm (G and I). Genotypes are *w*¹¹¹⁸ (A, B, and E–G) and *fab1*³¹/Df(2R)^{w30} (C, D, and H–J).

fab1 Mutant Cells Show Endosomal Accumulation of Receptors but Not of Ubiquitin

Because of the proposed relationship between endocytic trafficking and signaling (Lloyd *et al.*, 2002; Gonzalez-Gaitan, 2003; Piddini and Vincent, 2003), we asked whether endocytic trafficking and degradation of signaling proteins were

affected in *fab1* mutant polarized epithelial tissue. The receptors Notch (N) and PVR and the ligands Wingless (Wg), Delta (Dl), and Boss accumulated in punctate internal structures specifically in *fab1* mutant cells generated by the FLP/FRT technique (Golic, 1991; Xu and Rubin, 1993) or in dissected tissues from *fab1* mutants (Figure 4, A–F, K, and L;

Figure 4. Cell autonomous accumulation of receptors and ligands in *fab1* mutants. (A–C, and I) Wg is expressed in a central stripe of cells in the wing pouch and notum and taken up in neighboring cells where it can be observed in intracellular vesicles up to 20 μ m away from the source (I, projection of 50 confocal stacks 1 μ m apart). (D, E, G, H, and J) In *fab1* mutant wing discs, Wg accumulation can be seen in enlarged intracellular vesicles both in the wing pouch and the notum visible up to 40 μ m away from the source (J, projection like in I). This accumulation is specifically rescued in the wing pouch upon expression of full-length *fab1* in the wing pouch under *nub*-Gal4 control (G and G'), and not in the notum (H). (F) Cell autonomous accumulation of Wg is seen in *fab1* clones (dashed lines), labeled by the absence of GFP, outside the source of Wg production (overview shown in inset). (K) A similar accumulation of Notch in intracellular vesicles can be seen in *fab1* mutant cells of the eye disks posterior to the morphogenetic furrow (arrowheads). (L) A similar accumulation of the Notch ligand, Delta (DI), in *fab1* mutant follicle cells. (M and N) Ubiquitinated proteins accumulate in apical endosomes in *hrs* but not in *fab1* mutant clones (outlined by dashed lines) in the wing disk. Bars, 10 μ m (A, B, D, E, G, and H) and 50 μ m (I and J). Genotypes are *w¹¹¹⁸* (A–C and I), *fab1³¹/Df(2R)^{w30}* (D, E, and J), *fab1³¹,nub-Gal4/Df(2R)^{w30}; p [mini-w, UAS-fab1]/+* (G and H), *yw, hsp70-flp/+; FRT42D, p [mini-w, ubi-GFP]/FRT42D, fab1²¹* (F, K, L, and N), and *yw, hsp70-flp/+; FRT40A, p [mini-w, ubi-GFP]/FRT40A, hrs^{28D}* (M).

Figure 5. Receptors and ligands accumulate in enlarged late endosomal structures in *fab1* mutants. Endosomal trafficking of Wg in receiving cells was followed in control and *fab1* mutant wing disks. (A–D) Single confocal sections and separate channels of enlargement show localization of Wg in the lumen of enlarged Hrs-positive early endosomes and Rab7-positive MVBs in *fab1* mutant wing disks. (E and F) Wg colocalizes with 2xMYVE in both control and mutant cells (arrows). An additional pool of larger Wg-vesicles is prominent in mutant disks (arrowhead). (G and H) The largest Wg-positive structures (2–5 μ m) in mutant tissue colocalize with the luminal HRP moiety of the human Lamp1-HRP fusion protein, whereas little overlap is seen between Wg and HRP-Lamp1 in control tissue. (I) Endogenous Lamp1 is detected in punctae on the limiting membrane of the large Wg containing Lamp1-positive endosomes. (J) Like Wg, the large 2- to 5- μ m-sized Notch (N) containing structures contain Lamp1 in what seems to be the limiting endosomal membrane in *fab1* mutant clones (–/–), whereas little overlap is seen in heterozygous control cells (+/–). (K) The large Notch-containing structures colocalize with TRD. (L and M) Immunofluorescence of Wg and the HRP epitope in wing disks expressing Hrp-Wg under *dpp-Gal4* control (overview to the left and enlargement with overlay and separate channels to the right) shows extensive colocalization of Wg and HRP in large structures many cell diameters away from the source in mutant tissue. In control tissue, HRP is mainly detected alone in small structures. Genotypes are *w¹¹¹⁸* (A), *fab1³¹/Df(2R)^{w30}* (B, I, and K), *w¹¹¹⁸; pUAS-GFP-Rab7; dpp-Gal4/+* (C), *fab1³¹/Df(2R)^{w30}; pUAS-GFP-Rab7; dpp-Gal4/+* (D), *w¹¹¹⁸; pUAS-myc-GFP-dbFYVE/+; dpp-Gal4/+* (E), *fab1³¹/Df(2R)^{w30}; pUAS-myc-GFP-dbFYVE; dpp-Gal4/+* (F), *w¹¹¹⁸; dpp-Gal4/UAS-hrp-Lamp1* (G), *w¹¹¹⁸/+; fab1³¹/Df(2R)^{w30}; dpp-Gal4/UAS-hrp-Lamp1* (H), *yw, hsp70-flp/+; FRT42D, p [mini-w, ubi-GFP]/FRT42D, fab1²¹* (J), *w¹¹¹⁸/+; dpp-Gal4/UAS-hrp-Wg* (L), and *w¹¹¹⁸/+; fab1³¹/Df(2R)^{w30}; dpp-Gal4/UAS-hrp-Wg* (M). Bars, 5 μ m (A–H, and K), 2 μ m (I and J), and 1 μ m (L and M).

our unpublished data). Importantly, unlike *hrs* and *vps25* mutant cells, which showed dramatic accumulation of ubiquitin positive structures (Jekely and Rorth, 2003; Jekely *et al.*, 2005; Thompson *et al.*, 2005) (Figure 4M), *fab1* mutant cells did not accumulate any higher ubiquitin levels compared with neighboring control cells (Figure 4N). The strong accumulation of ligands and receptors but not of ubiquitin in *fab1* mutants suggests a distinct compartmentalization of cargo accumulation compared with the *tsg101*, *vps25*, and *hrs* mu-

mutants, in which ubiquitinated cargoes are retained in early Rab5/PtdIns(3)P-positive endosomes (Jekely and Rorth, 2003; Moberg *et al.*, 2005; Thompson *et al.*, 2005; Vaccari and Bilder, 2005).

fab1 Mutant Cells Contain Giant MVBs and Accumulate Receptors and Ligands in Late Endosomes

To characterize the trafficking/degradation defects in *fab1* mutants, we followed Wg trafficking and degradation in Wg

Figure 6. Trafficking and sorting of HRP-Lamp1 and HRP-Wg in *fab1* mutants. Electron micrographs showing localization of enzymatically converted HRP-Lamp1 and HRP-Wg expressed under *dpp-Gal4* control within the wing disk epithelium. (A and B) HRP-Lamp1 is found in typical MVBs in control wing disks (arrowheads in A). (C and D) In *fab1* mutant disks, HRP-Lamp1 accumulates in larger vesicles (arrowheads in C) of variable morphology. Some have irregular intraluminal vesicles of variable size (arrowhead in D), whereas others seem to be of more regular multivesicular morphology (arrow in D). (E and F) The intraluminal vesicles of MVBs in both wild-type (E) and *fab1* mutant cells (F) become more evident in tissue prepared by cryosectioning. (G and H) In control wing disks, HRP-Wg is mainly observed in MVBs ranging in diameter between 200 and 600 nm (our unpublished data). (I and J) In *fab1* mutant disks, HRP-Wg accumulates within much larger vesicles, indicating that ligand sorting from the limiting membrane had occurred. Genotypes are *dpp-Gal4/UAS-hrp-Lamp1* (A and B), *w¹¹¹⁸/+; fab1³¹/Df(2R)^{w30}*; *dpp-Gal4/UAS-hrp-Lamp1* (C and D), *w¹¹¹⁸* (E), *fab1³¹/Df(2R)^{w30}* (F), *w¹¹¹⁸*; *dpp-Gal4/UAS-hrp-Wg* (G and H), and *w¹¹¹⁸/+; fab1³¹/Df(2R)^{w30}; dpp-Gal4/UAS-hrp-Wg* (I and J).

receiving cells. Wg is secreted from a central stripe of cells in the wing pouch to the extracellular space and acts as a morphogen controlling wing margin specification and growth. In receiving cells of the wing disk, Wg is taken up by the Arrow/Frizzled2 receptor complex into the cells and degraded (Piddini *et al.*, 2005). Intracellular Wg in endocytic 0.5- to 1-μm-sized structures was recognized within receiving cells by immunolocalization up to 20 μm away from the source in both the wing pouch and the notum (Figure 4, A–C and I). Detection of Wg in isolated wing imaginal disks from *fab1* mutants revealed a striking accumulation of Wg in internal vesicles up to 40 μm away from the source of producing cells in the wing pouch (Figure 4, D, E, and J). The aberrant Wg accumulation was caused by the lack of Fab1, because expression of full-length Fab1 specifically in the wing pouch of *fab1* mutant animals showed a nearly complete rescue of the defect in the wing pouch (Figure 4G). The selective accumulation of Wg in receiving cells shows that the biosynthetic pathway is functional in *fab1* mutant cells. Moreover, Wg was specifically detected in *fab1* mutant cells

and not in heterozygous cells or wild-type twin spot cells located at a similar distance from the signaling source, suggesting that the excess of Wg protein in *fab1* cells derived from defective endocytic trafficking in the receiving cells (Figure 4F).

Comparison of confocal Z-sections revealed that although small 0.5- to 1.5-μm vesicles could be observed in the apical and basal portion of the receiving cells from both *fab1* mutants and control wing disks, the larger Wg-containing structures (up to 2–5 μm in diameter) were located further toward the perinuclear region (our unpublished data). Like in control cells (Figure 5, A, C, and E), Wg was observed in Hrs- and 2xFYVE (dbFYVE)-positive early endosomes as well as in Rab7-positive endosomes in *fab1* mutant tissue (Figure 5, B, D, and F). These endosomes seemed 1.5 to 2 times larger in diameter than in control cells and some contained Wg in the lumen, strongly suggesting that MVBs can form in *fab1* mutants and that Wg can be sorted to their inner vesicles. In control cells, Wg was rarely observed in GFP-Rab7- and GFP-2xFYVE-negative intracellular compartments, suggest-

Figure 7. Wg target gene activation in *fab1* and *hrs* mutant cells. Target gene expression activated by Wg signaling at different thresholds (summarized in I) was assessed in mosaic or mutant animals. (A–H and K) No discernible difference in target gene expression is observed in *fab1* mutant cells compared with control cells. (J) Like previously reported (Piddini *et al.*, 2005), *hrs* mutant cells display normal levels of Dll. Genotypes are *w¹¹¹⁸* (A–D), *fab1⁸/Df(2R)^{w30}* (E–H), *hsflp/+;FRT40A,Ubi-GFP/FRT40A, hrs^{28D}* (J), and *hsflp/+;FRT42D,Ubi-GFP/FRT42D, fab1³¹* (K).

ing that it is rapidly degraded after transport to the later lysosomal structures (Figure 5, C and E). The larger 2- to 5- μ m Wg-positive vesicles in *fab1* mutant cells were labeled by an antibody against the late-endosomal/lysosomal marker Lamp1 and contained an HRP-Lamp1 transgenic fusion protein (Figure 5, H and I). This suggests that although Wg traffics to late endosomes in *fab1* mutants, its degradation is inhibited.

Similarly to Wg, Notch was also observed in moderately enlarged Hrs-positive structures in *fab1* mutant cells (our unpublished data). The large vesicles containing Notch also displayed Lamp1 immunoreactivity, providing evidence that they represented late endosomes (Figure 5J). To verify that the enlarged structures accumulating Notch were indeed endocytic intermediates, we labeled late endosomes in *fab1* mutant eye disks by a 10-min uptake of TRD, followed by a 40-min chase period. TRD accumulated in large (2- to 5- μ m) structures frequently overlapping with Notch, suggesting that many of the large Notch-containing vesicles were endocytic (Figure 5K). These results indicate that Fab1 is required to establish or maintain the proper size of endosomal vesicles and for degradation of Wg and Notch, but not for trafficking of these proteins to late endosomes.

For a more detailed analysis, we expressed Wg and Lamp1 fused to HRP orthogonally to the endogenous Wg expression pattern in the wing disk by using the *dpp-Gal4* driver. The relatively high resistance of the HRP moiety of the fusion proteins to lysosomal degradation allows HRP signals to be detected in late endocytic compartments of receiving cells whose endogenous Wg is efficiently degraded, thus visualizing the entire endolysosomal system (Dubois *et al.*, 2001). Indeed, in control wing disks, immu-

nolocalization of HRP could be detected in numerous structures devoid of Wg (Figure 5L). In contrast, in *fab1* mutant disks, large structures, up to 5 μ m, filled with both HRP and Wg could be detected in receiving cells. This suggests that endolysosomal Wg degradation is inhibited in *fab1* mutant tissue (Figure 5M).

The HRP moiety of fusion proteins of HRP-Lamp1 and HRP-Wg is enzymatically functional in fixed tissue, allowing its in situ detection by EM after conversion of the diaminobenzidine substrate into a dark precipitate (Sevrioukov *et al.*, 1999; Dubois *et al.*, 2001). Consistent with previous findings (Sevrioukov *et al.*, 1999), we detected the HRP-Lamp1 fusion in MVBs by EM (Figure 6, A and B). Both normal-sized and enlarged MVBs containing HRP-Lamp1 in intraluminal vesicles could be observed in *fab1* mutant tissue, showing that the Hrs-dependent formation of intraluminal vesicles (Lloyd *et al.*, 2002) does not require Fab1 function (Figure 6, C–F). Although the MVBs in control cells were generally 0.5–1 μ m, the HRP-Lamp1-positive structures in *fab1* mutant cells were 1–5 μ m. These larger MVBs likely correspond to Lamp1/Wg/Notch-containing structures observed by confocal microscopy (Figure 5, I–K). Investigation of cells expressing HRP-Wg revealed abundant concentration of HRP in normal-sized endosomes of wild-type larvae (Figure 6, G and H) as well as in the larger structures in *fab1* mutants (Figure 6, I and J). In both cases, labeling was found both on limiting membranes and in intraluminal vesicles. Thus, both HRP-Wg and HRP-Lamp1 labeled similar MVBs and late endocytic compartments by EM. These results indicate that endocytosed Wg is transported into the lumen of MVBs and late endosomes in both wild-type and *fab1* wing disks.

Figure 8. Dpp and Notch target gene activation in *fab1* mutant cells. (A and B) The range of two cell rows with nuclear phosphorylated pMAD (arrowheads, optical Z-section; B) activated by Dpp in follicle cells from a wild-type egg chamber. (C and D) The range of pMAD activation is not changed in *fab1* mutant egg chambers (arrowheads, optical Z-section; D). (E and F) The same is true for *spalt* (Sal) activated in a gradient in response to Dpp signaling in the wing pouch (box in E enlarged in F). (G) No ectopic expression of the Notch target *Eyg* was observed in *fab1* mutant clones in eye disks. Genotypes are *w¹¹¹⁸* (A and B), *hsflp/+;FRT42D,Ubi-GFP/FRT42D, fab1³¹* (C–F), and *hsflp/+;FRT42D,Ubi-GFP/FRT42D, fab1²¹* (G).

Wg, Dpp, and Notch Signaling Is Normal in *fab1* Mutant Clones

Previous studies have shown that Hrs and ESCRT proteins are required for silencing of Dpp and Notch signaling, respectively (Jekely and Rorth, 2003; Moberg *et al.*, 2005; Thompson *et al.*, 2005; Vaccari and Bilder, 2005), whereas Wg signaling is unaffected in *hrs* mutants (Piddini *et al.*, 2005). Having observed that Wg and Notch trafficking is affected in *fab1* mutants, we therefore asked whether the impaired endocytic trafficking was associated with enhanced receptor signaling. The expression of Wg in the wing pouch activates target genes at distinct thresholds of signaling in a nested pattern away from the source and provides a sensitive read-out of signaling activity (schematically illustrated in Figure 7I). Investigation of mosaic or whole *fab1* mutant animals revealed no measurable defects on expression of the high-threshold target genes *Senseless* (Sens), *Cut*, and *Hindsight* (Hnt) or the low-threshold target gene *Distalless* (Dll) (Figure 7, A–K). This provides evidence that Wg signaling is unaffected in *fab1* mutants.

In *hrs* mutant cells, the Dpp receptor Thickvein accumulates in early ubiquitin-positive endosomes. This is associated with increased Dpp signaling activity in the wing pouch and follicle cells of the ovary (Jekely and Rorth, 2003).

In contrast, we did not detect any increase of the expression of the low level Dpp target gene *Spalt* (Sal) in *fab1* clones of the wing pouch, nor of phosphorylated Mothers against Dpp (p-Mad) in *fab1* clones of ovarian follicle cells (Figure 8, A–F). This indicates that Dpp signaling is unaffected in *fab1* mutants.

In *tsg101* or *vps25* mutant cells, Notch and Dll accumulate in an early endocytic compartment, leading to ectopic Notch signaling. In clones of the eye disk this results in the direct transcriptional activation of the downstream target genes, *unpaired* and *eyegone* (*eyg*) (Jekely and Rorth, 2003; Moberg *et al.*, 2005; Thompson *et al.*, 2005; Vaccari and Bilder, 2005). No ectopic expression of *Eyg* was observed in *fab1* mutant clones in eye disks (Figure 8G), suggesting that Notch signaling ensues at the correct space and time despite dramatic accumulation of both Dll and Notch in endosomes.

In the wing disk, short-range Notch signaling is instructive in defining the dorsal-ventral boundary organizer. A direct downstream consequence is the expression of Wg in a stripe of cells along the boundary. Thus, expression of Wg also serves as a marker for Notch signaling activity in the wing pouch (Vincent and Dubois, 2002). Even though the Wg stripe seemed broader than normal in *fab1* mutant disks, this was clearly due to accumulation of Wg in internal

endocytic vesicles and not due to a broader area of cells producing Wg, which are easily recognized due to large amounts of Wg in the biosynthetic pathway (our unpublished data). Moreover, no defects in the sensitive Notch-controlled bristle patterns and sensory organ cell specification in the cuticles of *fab1* mutants were observed (Figure 1, B and C). This supports the conclusion that Notch signaling is normal in *fab1* mutants. Collectively, the above-mentioned results indicate that Wg, Dpp, and Notch signaling is normal in *fab1* mutant cells. Thus, Fab1 does not share the ability of Hrs and ESCRT-I and -II to mediate receptor silencing.

DISCUSSION

PI kinases have crucial functions in cell biology and physiology and represent attractive targets for drugs against cancer and autoimmune diseases (Luo *et al.*, 2003; Murthy and De Camilli, 2003). Nevertheless, the functions of PtdIns(3)P 5-kinase in multicellular organisms have not been investigated. In the present study, we have established Fab1 as an essential PtdIns(3)P 5-kinase in *Drosophila*, with profound functions in membrane traffic. Although the importance of Fab1 in endocytic membrane homeostasis has previously been highlighted by studies in yeast and mammalian cell culture (Odorizzi *et al.*, 1998; Ikononov *et al.*, 2001, 2003; Bonangelino *et al.*, 2002), its failure to mediate receptor silencing was unanticipated. Our data place Fab1 downstream of Hrs and ESCRT-I and -II, which are known to mediate receptor silencing in endosomes.

That endosomal sorting of ubiquitinated cargoes is of great physiological importance is illustrated by studies of *Drosophila* mutants of the two ESCRT subunits, Tsg101 and Vps25. Loss of these proteins yields endosomal accumulation of receptors and ubiquitin, similarly to *hrs* mutants. Importantly, loss of Tsg101 and Vps25 in clones of cells causes a tumor-like overproliferation of adjacent tissue due to increased Notch-mediated signaling (Moberg *et al.*, 2005; Thompson *et al.*, 2005; Vaccari and Bilder, 2005). We were unable to detect any such effects with *fab1* mutant clones (our unpublished data) consistent with the finding that Notch signaling (as well as Wg and Dpp signaling) was unaffected in *fab1* mutants. Thus, Fab1, unlike Hrs and ESCRT-I and -II proteins, does not seem to play any role in receptor silencing, even though it is important for receptor degradation. This is reminiscent of the ESCRT-III subunit hVps24, which mediates degradation but not silencing of the epidermal growth factor receptor (Bache *et al.*, 2006). Moreover, it is interesting to note that impaired Hrs, Tsg101, or Vps25 function causes a strong accumulation of ubiquitinated proteins in endosomes (Bishop *et al.*, 2002; Jekely and Rorth, 2003; Thompson *et al.*, 2005), whereas this was not observed in *fab1* mutant clones. These results, together with the fact that Fab1 mainly localizes to later endocytic structures than Hrs, suggest that Fab1 functions later than Hrs and ESCRT-I/-II in endocytic trafficking, at a point beyond receptor deubiquitination and signal termination.

Studies in yeast and mammalian cells have suggested a role for Fab1 in endocytic membrane homeostasis, although its exact functions are not known. Indeed, confocal and EM revealed the accumulation of larger late endosomes in *fab1* mutant *Drosophila* cells, consistent with previous studies in *fab1* yeast and overexpression of kinase-dead PIKfyve in mammalian cells. The findings that the enlarged vacuoles in *fab1* yeast mutants and late endosomes in kinase-dead PIKfyve-overexpressing cells contain few internal vesicles have suggested the possibility that Fab1 could mediate formation

of such vesicles (Odorizzi *et al.*, 1998; Ikononov *et al.*, 2001). In agreement with this, in *fab1* mutant *Drosophila* cells we frequently observed enlarged endosomes with few or no intraluminal vesicles. However, in the *fab1* mutants we also observed highly enlarged MVBs that were filled with numerous normal-sized intraluminal vesicles. This indicates that the increased endosome size in the absence of Fab1 cannot be explained by an inhibited formation of intraluminal vesicles, in contrast to what has been reported for Hrs (Lloyd *et al.*, 2002). A more likely explanation is that late endosomes expand in *fab1* mutants because of inhibited retrograde membrane flux to the biosynthetic and early endocytic pathways (Dove *et al.*, 2004; Efe *et al.*, 2005).

Cell and organ size is controlled by genetic, hormonal, and environmental inputs (Hafen and Stocker, 2003). In particular, insulin signaling is important for growth, and the functions of the downstream class I PI 3-kinases in growth signaling are well characterized. The striking growth phenotypes observed in *fab1* mutants indicate that PtdIns(3)P 5-kinase also regulates cell size. Interestingly, however, whereas PI 3-kinases promote growth, our findings indicate that Fab1 has an inhibitory effect on cell size. Garland cells were strongly enlarged in *fab1* mutants, suggesting a function of Fab1 in negative cell size regulation. In addition, *fab1* deficiency led to a thickening of legs and enlargement of wings and heads, demonstrating a role for Fab1 in attenuating organ size. Overexpression of *Drosophila* Fab1 did not cause any overt growth-inhibitory effects (our unpublished data), consistent with the finding that overexpression of Fab1 in yeast does not yield any increase in PtdIns(3,5)P₂ levels, presumably because regulatory components are limiting (Odorizzi *et al.*, 1998). We were unable to detect any strong genetic interactions between *fab1* and mutants in components of the insulin signaling pathway (our unpublished data), suggesting that the increased cell size in *fab1* mutants may not be due to up-regulation of this pathway. Instead, there was a striking correlation between cell size and endosome overgrowth in *fab1* mutant larvae. Thus, the increased cell and organ size in *fab1* mutants may be due to the volume expansion of endosomes. We therefore propose that Fab1, through its effects on endosome morphology, functions in negative regulation of cell volume. Further work will reveal whether Fab1 also regulates cell size by additional mechanisms.

In conclusion, we have performed the first analysis of a PtdIns(3)P 5-kinase in a multicellular organism and found that Fab1 is essential for proper degradative receptor trafficking but dispensable for the termination of receptor signaling. This places the function of Fab1 at a late stage in endocytic receptor trafficking and shows that receptor degradation is not required for signal termination.

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