Characterisation of the phosphatidylinositol-3-phosphate 5-kinase, Fab1, in *Drosophila melanogaster*

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In the memory of “pappa”

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Abbreviations

DEP	Dishevelled, EGL-10, Pleckstrin domain
EE	Early endosome
EGFR	Epidermal growth factor receptor
EMS	Ethylmethane sulfonate
ER	Endoplasmic reticulum
ESCRT	Endosomal sorting complex required for transport
Fab1	Fragmented and binucleated 1
FYVE	PI(3)P binding domain conserved in Fab1, YOTB, Vac1, EEA1
Hrs	Hepatocyte-growth-factor-regulated tyrosine-kinase substrate
LE	Late endosome
MVBs	Multivesicular body
OGD	Oregon green dextran
PH	Pleckstrin homology
PI	Phosphoinositide
PI(3)P	Phosphatidylinositol-3-phosphate
$PI(3,4)P_2$	Phosphatidylinositol-3,4-bisphosphate
$PI(3,4,5)P_2$	Phosphatidylinositol-3,4,5-trisphosphate
$PI(3,5)P_2$	Phosphatidylinositol-3,5-bisphosphate
PI(4)P	Phosphatidylinositol-4-phosphate
$PI(4,5)P_2$	Phosphatidylinositol-4,5-bisphosphate
PI(5)P	Phosphatidylinositol-5-phosphate
PI3K	Phosphatidylinositol-3-kinase
PI3P-5K	Phosphatidylinositol-3-phosphate-5-kinase
PI4K	Phosphatidylinositol-4-kinase

PI5K	Phosphatidylinositol-5-kinase
PIKfyve	Phosphoinositide kinase with specificity for the five positions containing a fyve finger
PIK	Phosphatidylinositol-kinase
PtdIns	Phosphatidylinositol
PX	Phox homology
Snf7	Sucrose non-fermenting 7
STE3	Pheromone a factor transmembrane receptor in *Saccharomyces cerevisiae*
TGN	Trans Golgi network
Tsg101	Tumour susceptibility gene 101
UIM	Ubiquitin interacting motif
Vps	Vacuolar protein sorting

Aim of the present study

Receptor downregulation is an important cellular function. Growth factor receptors are downregulated by uptake in the endocytic pathway and subsequent degradation in the lysosomes. Defective receptor downregulation may cause uncontrolled cell proliferation and cancer. Characterisation of the molecular mechanisms concerning receptor downregulation is therefore important. Phosphoinositides (PIs) are lipids known to regulate receptor downregulation. Previously it has been established that phosphatidylinositol-3-phosphate (PI(3)P) regulates endocytic membrane trafficking by recruiting intracellular effectors containing a PI(3)P binding FYVE (conserved in Fab1, YOTB, Vac1, EEA1) domain. The yeast FYVE domain-containing kinase, Fab1 (fragmented and binucleated 1), is involved in membrane trafficking and receptor sorting into the multivesicular bodies (MVBs). The receptors are degraded upon fusion with the vacuole (equivalent to the mammalian lysosome) and thereby downregulated. *FAB1* is highly conserved in higher organisms and we hypothesise a similar function of the kinase in flies and humans. The aim of this project was to take advantage of the genetic tools available in the model organism *D. melanogaster* in order to characterise the *FAB1* homologue and its function in endocytic trafficking and cell signalling. Information obtained from the fruit fly will be highly relevant in humans due to the conservation of the kinase throughout evolution.

1. Introduction

Endocytosis and vesicle trafficking play crucial roles in cell function by regulating cell-cell communication and signalling. Cell growth, differentiation and proliferation are stimulated by activated receptors, such as the epidermal growth factor receptor (EGFR), at the cell surface. This receptor is downregulated by endocytosis, sorted into multivesicular bodies (MVBs) and subsequently degraded in the lysosomes. PI kinases (PIKs) and their products PIs are important components of this machinery. Defective degradation of activated receptors can lead to uncontrolled cell proliferation and cancer (Katzmann et al., 2002; Raiborg et al., 2003). By investigating the endocytic sorting machinery of the cell, one can understand the molecular mechanisms behind serious diseases and use this knowledge to design therapeutic drugs. A useful model to investigate these processes *in vivo* is the fruit fly, *D. melanogaster*. This thesis concerns investigation of the PIK, Fab1, in *D. melanogaster* and its potential role in regulating endocytic trafficking.

1.1 Endocytosis and vesicle trafficking

In all eucaryotic cells, membrane trafficking and cargo sorting are essential processes. The membrane traffic flows along highly organised directional routes. The eucaryotic cells have evolved a system of internal membranes, the **biosynthetic pathway,** transporting newly synthesised carbohydrates and lipids to target compartments. The transport of the biosynthetic pathway goes from the endoplasmic reticulum (ER) toward the Golgi apparatus and then either out of the cell or to the lysosome. This pathway allows the cell to modify the molecules produced through a series of steps, store them until needed, and then secrete them to the exterior of the cell in a process called **exocytosis**. The transport and storage of these molecules also takes place in conjunction with the second major vesicle transport system, the **endocytic pathway**. This system of internal membranes allows the cells to take up macromolecules from

the outside by a process known as **endocytosis** (Figure1A) (Sanderfoot and Raikhel, 1999).

The endocytic pathway transports, among other proteins, transmembrane proteins from the plasma membrane to the early endosomes (EEs) and late endosomes (LEs)/ MVBs (referred to as MVBs hereafter). Certain transmembrane proteins in the early endosomes are targeted for degradation in the lysosome via budding of inner vesicles into the MVBs. Proteins, which are embedded in the membrane of inner vesicles, are released and degraded upon fusion between the lysosomal- and the MVB-limiting membranes by lysosomal proteases, lipases and other hydrolases (Katzmann et al., 2002). In both endocytic and biosynthetic trafficking the flow of membrane compartments is strictly regulated and balanced. The flow in opposite directions brings membrane and selected proteins back to the compartment of origin. Each transport vesicle needs to be very selective to perform this task; it must fuse only with the appropriate target membrane and take up only the correct proteins. A great deal is known about the molecular mechanisms of endocytic sorting, as outlined below.

1.1.1 MVB; A molecular sorting station

Fifty years ago the first MVB was described by electron microscopy as a spherical organelle of around 400-500 nm with a limiting membrane enclosing several internal vesicles of 40-90 nm (Katzmann et al., 2002). MVBs represent endocytic intermediates formed from EEs (Raiborg et al., 2003). The MVBs serve as sorting stations and receive cargo from the endocytic pathway and the biosynthetic pathway. The sorting of cargo into intraluminal vesicles has several important functions. First, transmembrane proteins that are to be released from the cell in a regulated manner might be stored in intraluminal vesicles and the MVB might function as a vehicle for this matter. Second, the proteins in the MVB limiting membrane are usually resistant to degradation by proteases upon fusion with the lysosomes, because they only expose their luminal region that is usually extensively glycosylated. However, the proteins

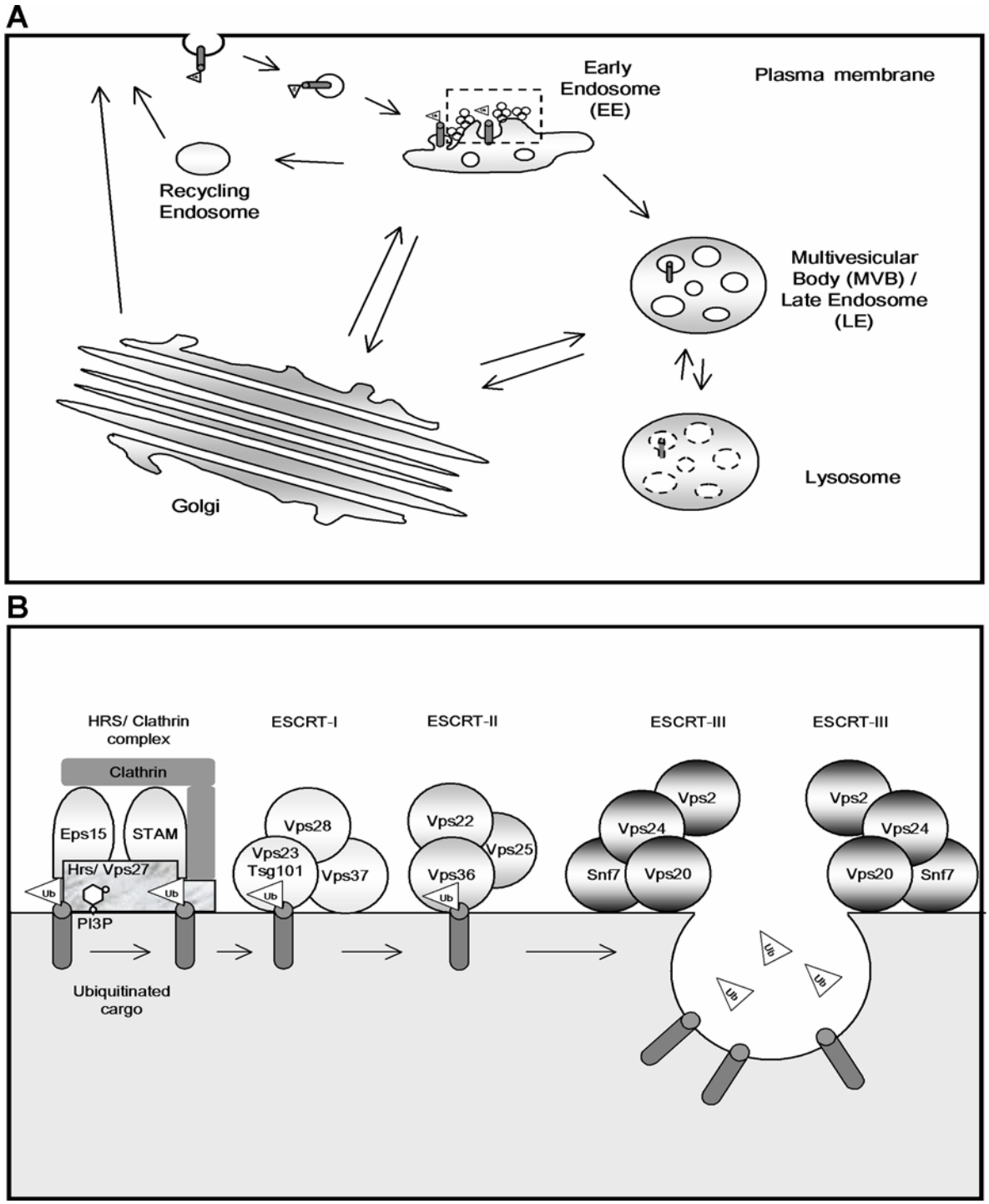

Figure 1 Model for the endocytic pathway and protein sorting into the MVB. **(A)** The endocytic pathway transports, among other proteins, transmembrane proteins, from the plasma membrane to the EE and LE/ MVBs for degradation in the lysosomes. The sorting of cargo into intraluminal vesicles has several important functions, such as signal downregulation. The EGFR, for example, is monoubiquitinated at multiple sites, and this modification is interpreted as a sorting signal into the inner vesicles of MVBs. The area indicated by a rectangle is enlarged in (B). **(B)** Together with monoubiquitin, other protein complexes, such as the ESCRTs, are involved in the formation of the MVB inner vesicles and thereby the sorting process. The lipid PI(3)P mediates localisation of the FYVE-domain containing protein Hrs and its associated proteins to the endosomal membranes. This complex binds to ubiquitinated protein and retains them in the membrane. Hrs recruits ESCRT-I by interacting with Tsg101. The ubiquitinated protein is relayed on to ESCRT-II and sorted into the MVB by polymerisation of ESCRT-III. The arrows indicate the direction of the sorting process. (The figure is adapted from (Raiborg et al., 2003)).

sorted into the intraluminal vesicles of the MVBs will be degraded by lysosomal hydrolases. Third, MVBs can play an important role in signal downregulation: In principle, signalling from internalised receptors is possible from the limiting membrane of MVBs (Lloyd et al., 2002). The ability of endocytosed receptors, such as the EGFR, to transmit signals inside the cell is believed to be blocked by enclosing them in intraluminal vesicles of MVBs (Raiborg et al., 2003). The activated EGFR associates with several key components of the signalling transduction machinery. By sorting it into the intraluminal MVB vesicles its cytoplasmic domain is segregated away from the cytoplasm, and thereby interaction is prevented (Katzmann et al., 2002). In mice, mutations interfering with the MVB sorting of EGFRs lead to tumorigenesis (Ceresa and Schmid, 2000; Di Fiore and Gill, 1999), and in *D. melanogaster* such mutations cause prolonged growth-factor-stimulated signalling and embryonic patterning defects (Lloyd et al., 2002).

1.1.2 Molecular mechanisms of protein sorting into MVBs

To understand the downregulation of transmembrane receptors it is necessary to define the proteins that target them to the intraluminal vesicles of MVBs (Raiborg et al., 2003). By studying the molecular mechanisms behind trafficking of the transmembrane receptor EGFR, it is possible to obtain general information about the principles behind endocytotic sorting and degradation (Felder et al., 1990). The lipid PI(3)P is to be found on EE membranes (Gillooly et al., 2000). The PI(3)P binding protein Hrs (hepatocyte-growth-factor-regulated tyrosine-kinase substrate) together with monoubiquitination of target proteins has been shown to serve as a mediator of MVB-sorting (Hicke, 2001; Marchese and Benovic, 2001; Rocca et al., 2001; Rotin et al., 2000; Shenoy et al., 2001). Monoubiquitination of proteins from the Golgi, plasma membrane and endosomes has been shown to target them to the lysosome (Raiborg et al., 2002; Reggiori and Pelham, 2001; Urbanowski and Piper, 2001). The EGFR is monoubiquitinated at multiple sites, and this modification is interpreted as a sorting signal to target them to the inner vesicles of MVBs. Together with Hrs and

monoubiquitin, other protein complexes are involved in the formation of the MVB inner vesicles and thereby the sorting process. This intricate system is outlined below.

The lipid modifying enzyme, PI 3-kinase (PI3K), modifies the membrane lipid PtdIns to PI(3)P (the PIs and PIKs are dealt with in detail later) (Fruman et al., 1998). Inhibition of PI3K by wortmannin or an antibody prevents the formation of intraluminal vesicles in MVBs (Fernandez-Borja et al., 1999; Futter et al., 2001). Likewise, sequestration of endosomal PI(3)P by a tandem PI(3)P binding FYVE domain inhibits the sorting of activated EGFRs into MVBs, but transport of other cargos between the early and late endosome is not affected (Petiot et al., 2003). This indicates that PI(3)P is not required for the formation of MVBs as such, but is required for the formation of intraluminal vesicles in MVBs and sorting of EGFR to these vesicles by recruiting PI(3)P binding effector proteins. Many of the proteins that bind PI(3)P do so either through a FYVE domain or a PX domain (Phox homology domain), and there are around 70 different proteins containing one of these domains in mammals (Gruenberg and Stenmark, 2004). The lipid PI(3)P mediates localisation of the FYVE-domain containing protein Hrs and its associated proteins to the endosomal membranes (Raiborg et al., 2001). Hrs has a ubiquitin-interacting motif (UIM), that is able to directly bind ubiquitinated proteins (Raiborg et al., 2002). The function of the Hrs -protein complex is believed to be to bind to ubiquitinated membrane proteins in order to prevent them from recycling back to the plasma membrane (Raiborg et al., 2003). Direct evidence for the role of Hrs in lysosomal targeting of ubiquitinated proteins comes from studies in *Drosophila melanogaster*, in which *hrs* mutants display enlarged endosomes, a reduced number of intraluminal vesicles in MVBs and increased EGFR signalling. This shows that Hrs is required for the sorting of EGFRs into the inner vesicles of MVB and their subsequent degradation (Lloyd et al., 2002).

Many of the components in the endocytic sorting machinery, such as the ESCRTs (endosomal sorting complexes required for transport), have ubiquitin-binding domains (Katzmann et al., 2002). The ESCRTs consist of several vacuolar-sorting

proteins (Vps). Hrs is indirectly involved in the formation of the intraluminal vesicles in MVBs by recruiting ESCRTs to the endosomal membranes (Bache et al., 2003; Katzmann et al., 2003) (Figure 1B). The Hrs protein complex is believed to deliver its ubiquitinated proteins to ESCRT-I, a complex consisting of three subunits (Vps23, Vps28 and Vps37) by binding the subunit Vps23 in yeast (Tsg101 (Tumor susceptibility gene 101) in mammalian cells) which also contains a ubiquitin binding domain (Bache et al., 2003; Katzmann et al., 2003; Lu et al., 2003; Pornillos et al., 2003). Interaction between Hrs and ESCRT-I is important for the sorting of ubiquitinated cargo to the degradation pathway (Katzmann et al., 2003; Lu et al., 2003). The ubiquitinated protein is further relayed on to another three-subunit complex, the ESCRT-II (Vps22, Vps25 and Vps36). The ESCRT–II subunit, Vps36, contains a ubiquitin binding-domain which is believed to have a function in the sorting of ubiquitinated proteins into endosomal invaginations. The ubiquitinated protein is transported into an intraluminal vesicle, whose formation requires the polymerisation of a third multisubunit complex, the ESCRT–III (Vps2, Snf7, Vps20 and Vps24) on the endosomal membrane (Raiborg et al., 2003). *Saccharomyces cerevisiae* cells that lack one or more of the ESCRT-subunits have defective protein sorting to the vacuole and do not form the intraluminal vesicles (Katzmann et al., 2003).

Another protein containing a FYVE-domain, like Hrs, is the *S. cerevisiae* phosphatidylinositol-3-phosphate 5-kinase (PI3P-5K) Fab1 and its mammalian homologue PIKfyve. This kinase is localised to endosomal membranes (Sbrissa et al., 2002). In mammalian cells, overexpression of a dominant negative PIKfyve does not lead to elimination of the inner vesicles of MVBs, indicating that the PI3P-5K is not the only protein needed for the intraluminal vesicle formation. However, Fab1 might play an important role in the sorting of a subset of proteins into the intraluminal vesicles (Reggiori and Pelham, 2002). The ESCRT-III complex contains a subunit, Vps24, that is able to bind $PI(3,5)P_2$, the catalytic product of Fab1. This finding suggests that $PI(3,5)P_2$ might function as an activator of ESCRT-mediated protein sorting (Whitley et al., 2003).

1.1.3 Phosphoinositides and lipid kinases

PIs are phosphorylated derivates of phosphatidylinositol (PtdIns), a lipid with two fatty acid tails and an inositol head group. PtdIns is a unique component among phospholipids in the eucaryotic cell membranes in the sense that its head group can be phosphorylated at free hydroxyls (Figure 2). Site- and time specific PI production has been found to play important roles in many cellular responses. PIs are involved in many cellular processes, such as proliferation, survival, cytoskeletal organization, glucose transport, platelet function and vesicle trafficking. The enzymes that phosphorylate PtdIns and its derivates are the PIKs (Fruman et al., 1998).

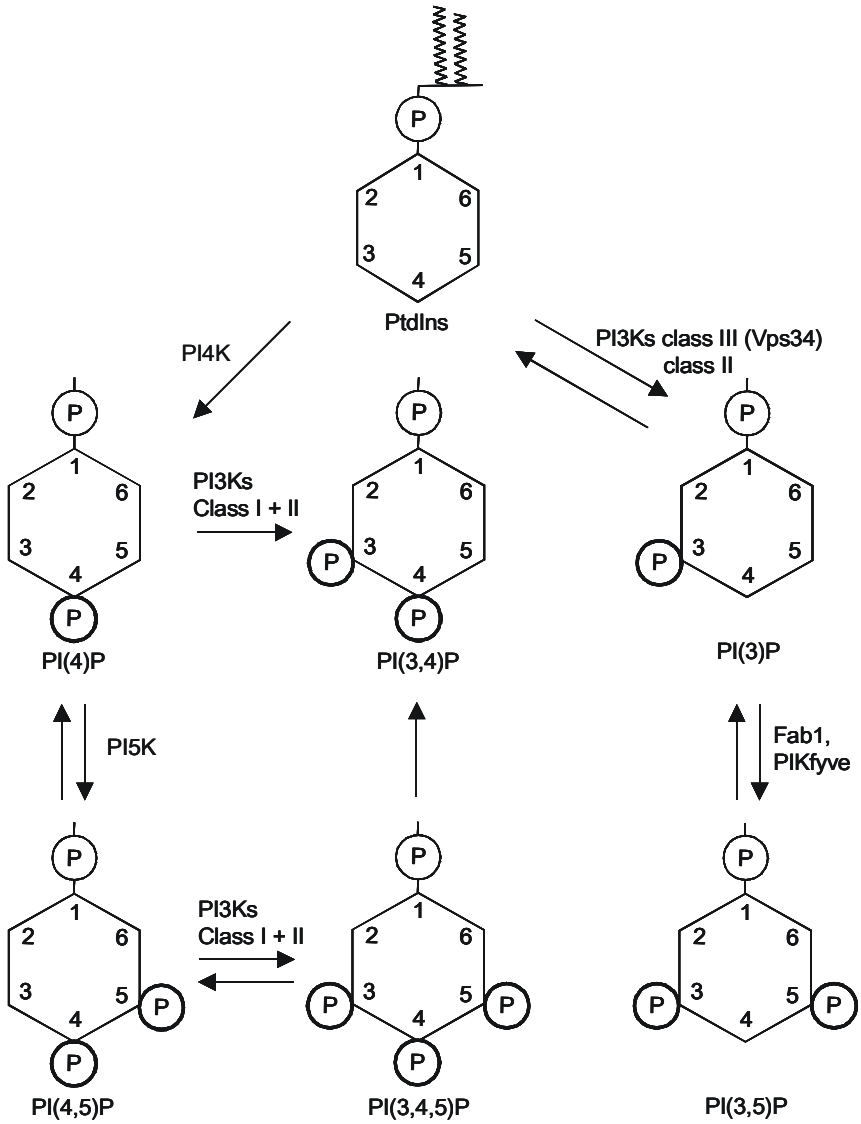

Figure 2 Phosphoinositides (PIs). PIs are phosphorylated derivates of phosphatidylinositol (PtdIns). The inositol ring can be phosphorylated in position 3, 4 and 5 intracellularly by PIKs. The PIKs are indicated in-between their corresponding substrate and product. Phosphatases are not included. In cells, the following PIs have been identified; PI(3)P, PI(4)P, PI(5)P, $PI(3,4)P_2$, $PI(3,5)P_2$, $PI(4,5)P_2$ and $PI(3,4,5)P_3$. PI(5)P is not included in the figure because its function is unknown. (The figure is adapted from (Simonsen et al., 2001)).

Positions 3, 4 and 5 in the inositol ring are the only ones found to be phosphorylated intracellularly (Shisheva, 2001). The ring can be phosphorylated in these positions separately or in all possible combinations. In cells, the following PIs have been identified; PI(3)P, PI(4)P, PI(5)P, PI(3,4)-bisphosphate (bisphosphate termed P_2 hereafter), PI(3,5)P_2, PI(4,5)P_2 and PI(3,4,5)-trisphosphate (trisphosphate termed P_3 hereafter). In unstimulated eucaryotic cells, PtdIns is the most abundant phospholipid and the others are present in much lower amounts (Table 1) (Stephens et al., 2000). Although the PIs are present as a small fraction of the total cellular phospholipids, they play a crucial role in signal transduction as the precursors of several second-messenger molecules. The PI lipids could be imagined to have several different functions in the cell: (1) alteration of local membrane topology by electrostatic interactions, (2) direct interaction with intracellular proteins and thereby affect their localisation and/ or activity, (3) function as phospholipase substrates for soluble inositol phosphate (inositol(1,4,5) trisphosphate (IP_3) and membrane-associated diacylglycerol) second messengers (Fruman et al., 1998).

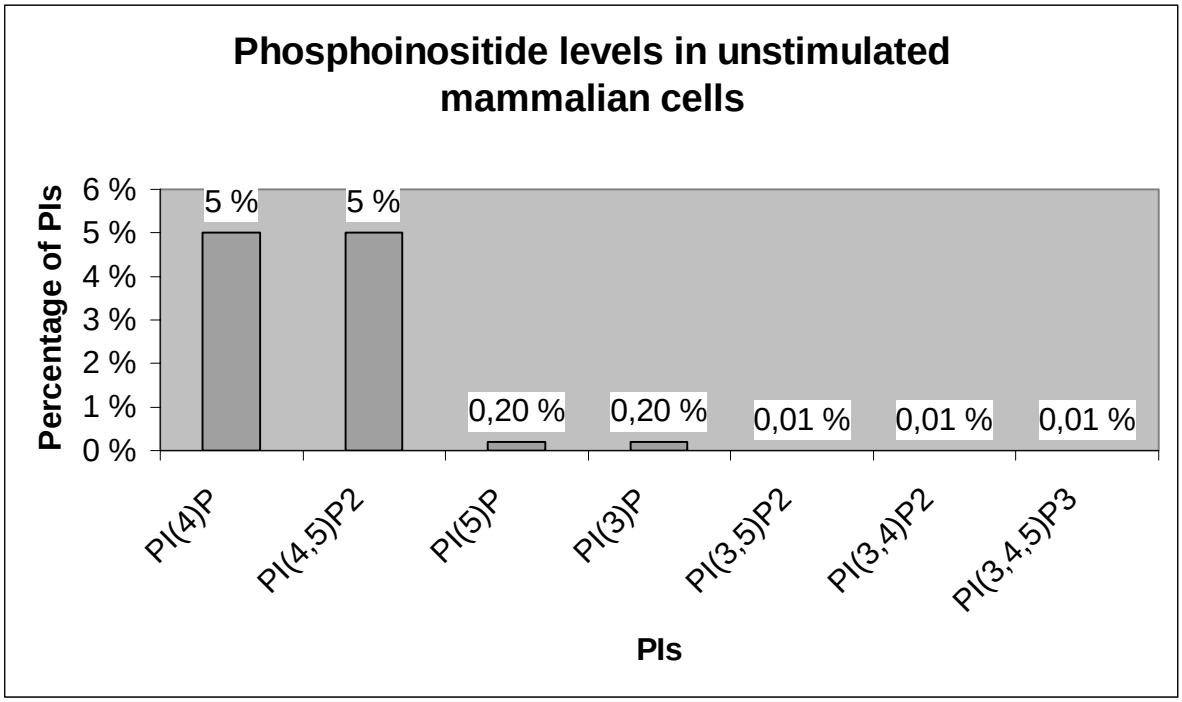

Table 1 Phosphoinositide levels in an unstimulated mammalian cell. The table shows approximate levels of PIs. The most abundant lipid, PtdIns, is set to 100%. The levels of PI(4)P and PI(4,5)P_2 are around 5%, followed by PI(5)P, PI(3)P at 0,2% and PI(3,5)P_2, PI(3,4)P_2 and PI(3,4,5)P_3 at 0,01%. (The figure is adapted from (Stephens et al., 2000)).

PI kinases

PIs are mainly synthesised by lipid kinases, although some PIs can be generated by lipid-specific phosphatases acting upon more highly phosphorylated forms (the phosphatases are not going to be dealt with here). The PIKs are divided into different classes based on the enzymatic activity leading to transfer of a phosphate to a specific position in the inositol ring. Three general families were generated on the basis of this, PI3Ks, PI4Ks and PI5Ks. The PI4Ks are not going to be dealt with here. Lower eucaryotes and yeast express kinases with substantial protein-sequence homology to their mammalian counterparts, and members of each kinase family have been identified. Support to the classification of separate families is given by the finding of sequence homology, and the importance of these enzymes is underscored by their conservation throughout evolution (Fruman et al., 1998).

PI3Ks

Studies of PI3Ks have revealed a role both in growth regulation and various other cellular responses (Franke et al., 1997). The PI3Ks are divided into three classes, class I, -II and –III, according to their recognised substrate, but all three classes phosphorylate the inositol ring in the third position. Class I and II are not going to be dealt with here.

The class III PI3K was identified in a screen for mutant yeast cells defective in vacuolar protein sorting (Herman and Emr, 1990). When the corresponding gene *VPS34,* was cloned, it was found to be essential for accurate transport of newly synthesised proteins from the Golgi apparatus to the vacuole (Schu et al., 1993). Class III PI3Ks only phosphorylate PtdIns to produce PI(3)P (Figure 2) (Fruman et al., 1998). These PI3Ks do not appear to be acutely regulated by cell-surface receptors, but rather in agonist-independent membrane trafficking (Toker et al., 1995).

The PI3P-5K, Fab1

From the cellular pool of PI(3)P, PI(3,5)P_2 is synthesised by a PI3P-5K. In humans a PI3P-5K, PI5Kα, has been cloned and the kinase domain is related to a protein in *S. cerevisiae,* Fab1. In resting mouse cells PI(3,5)P_2 is a product of an agonist-independent pathway and their relatively constant cellular levels are maintained by a reciprocal action between PI(3,5)P_2 5-phosphatase (not dealt with here) and PI3P-5K called PIKfyve (Figure 2) (Whiteford et al., 1997).

FAB1 (yeast) and PIKfyve (mouse) are orthologs of a PI3P-5K that belongs to an ancient gene family. The gene is conserved in many different species such as mouse (*mus muculus*), human (*Homo sapiens*), yeast (*Saccharomyces cerevisiae, Schizosaccharomyces pombe*), and *Caenorhabditis elegans, Arabidopsis thaliana* and the fruit fly (*Drosophila melanogaster*) (Shisheva, 2001). The yeast gene, *FAB1*, was first discovered in a screen for defects in protein sorting. The yeast cells had enlarged, deacidified vacuoles and the cell membrane receptor, STE3, failed to be sorted into the vacuolar lumen. Instead the receptor was missorted to the outer membrane of the vacuole. The FM4-64 dye inserts into the plasma membrane and when the membrane is internalised by endocytosis, the dye will label the membranes of endocytic intermediates, the internal vesicles of MVBs and eventually the vacuole membrane. The delivery of the lipophilic fluorescent dye FM4-64 from the plasma membrane to the vacuole, is delayed in the yeast *fab1* mutants compared to wild type cells (Shaw et al., 2003). It has been observed that mammalian cells expressing a kinase defective mutant of PIKfyve have altered morphology. The cells show enlarged endocytic vesicles, indicating that PIKfyve enzymatic activity plays a crucial role in regulating membrane trafficking. It still remains to be directly tested whether the PI(3,5)P_2 product of PIKfyve gives the specific phenotype. However it is likely that the effect is due to loss of PI(3,5)P_2, as observed in yeast cells with an inactive *FAB1* gene (Odorizzi et al., 2000). Studies of PIKfyve at the protein level have revealed a widespread distribution of the protein among cells and tissues (Sbrissa et al., 2000). Immunofluorescence microscopy in 3T3-L1 adipocytes (a fat storage cell in mammals) has found the endogenous protein distributed in the cells periphery and

excluded from the nucleus. Biochemical analysis has revealed that around 76% of the protein can be detected in the cytosol, 20% associated with intracellular membranes and 4% with the plasma membrane. Analysis of the intracellular membrane fraction has suggested that PIKfyve associates with the membranes of the late endocytic pathway (Shisheva et al., 2001). Late endosomal PI(3)P is probably both a binding site for the FYVE-domain in PIKfyve/ Fab1 and a source of PI(3,5)P_2 production (Sbrissa et al., 2002).

To be able to investigate the role of Fab1 in membrane trafficking and cell signalling in a multicellular system, and not only in a single cell, *Drosophila melanogaster* was chosen as a model system.

1.2 The model organism *Drosophila melanogaster*

Since Thomas Hunt Morgan decided to use *D. melanogaster* as an organism to investigate the chromosomal theory of inheritance at the beginning of the last century, it has been one of the favourite model organisms of geneticists (Weiner, 1999). The reason why Morgan chose *D. melanogaster* was because it is cheap to keep in the laboratory, has a short generation time (only ten days) and produces many progeny. There are, however, other advantages to working with this model organism. The fruit fly has only four chromosomes and there is no meiotic recombination in males, making it relatively easy to track chromosomes through generations. The giant polythene chromosomes of the larval salivary glands formed by many rounds of DNA replication align tightly side by side in a parallel register. This can be visualised by a normal light microscopy and the structure of the chromosomes can thus easily be determined making it possible to probe genes and position them on the chromosome. The development of the external features of the fly, such as wings, bristles and compound eyes can be affected by mutations. Simply by looking at the fly in the stereomicroscope, one can spot phenotypic mutants arising from genomic mutations. Combining these features, it is possible to study the molecular and genetic basis of a phenotype. Succeeding drosophilists have developed several sophisticated techniques

that make the fruit fly one of the best model organisms for genetic analysis of almost any process (Rubin and Lewis, 2000).

Even though insects and vertebrates diverged around 700 million years ago, flies represent organisms that are surprisingly similar to vertebrates with respect to developmental processes (Adams et al., 2000). A surprisingly large number of the approximately 15.000 *D. melanogaster* genes have proven to have overt human homologues (Friedman and Hughes, 2001). Out of the 287 known human disease genes, 197 have homologues in *D. melanogaster* and produce very similar symptoms in flies when mutated. Even the human disease genes that have no fly homologue produce similar symptoms to those observed in humans when expressed in *D. melanogaster* (Feany and Bender, 2000; Fortini et al., 2000). The ability to carry out large-scale genetic screens for mutations affecting a given process is one of the most important features that *D. melanogaster* provides. The gene affecting the process can be identified and at the same time the fly serves as a very practical tool for genetic investigation (St Johnston, 2002).

1.2.1 The life cycle of *Drosophila melanogaster*

In mammals, the fertilized embryo develops inside the mother's uterus and is born as a miniature version of the adult. *D. melanogaster* has a different life cycle. The fly goes through four stages; embryo, larvae, pupa and adult fly (Figure 3). The fertilized embryo is laid in nutritious food, such as bananas or other types of fruits. After one day, when the larva hatches, it is surrounded by nutrition. The larva spends almost all its time eating and gaining weight to expand its tissues. When the larva grows, the skin becomes too small. There are three larval stages (instars) separated by moults where the skin is shed. At the end of the third instar, about five days after fertilisation of the embryo, the larva stops feeding, moults one more time and forms an immobile pupa. In this pupal stage the larva goes through complete metamorphosis where all the larval tissues are reorganised to form the adult fly. The whole process takes approximately ten days.

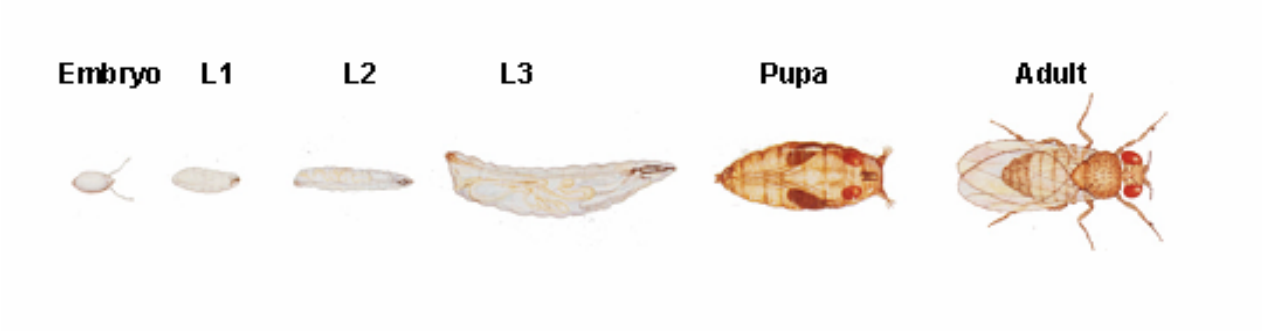

Figure 3 Life cycle of *D. melanogaster*. The larva hatches from the embryo and goes through three larval stages (instars) called L1, L2 and L3 as it grows. At the end of the third instar, L3, the larva becomes immobile and forms a pupa. In the pupa the larvae goes trough total metamorphosis and developes into a fly. The whole process takes 10 days. (The figure is adapted from http://www5.indire.it:8080/set/biotecnologie/drosophila/dros3.htmdrosophila/dros3.htm).

1.2.2 Embryonic and imaginal disc development

Large amounts of maternal mRNAs and proteins are deposited in the eggs of insects, before the embryonic nuclei starts to function, to facilitate rapid development. In *Drosophila* these proteins and mRNAs are produced in the follicle and nurse cells and thus come from the genome of the mother fly. These genes are called “maternal contribution genes” and consist of “housekeeping genes” necessary for basic cell function, early development and patterning. The scientific disadvantage with this maternal mRNA contribution is that the effect of mutated genes on development and embryonic patterning can be masked by the presence of the maternal protein product (Perrimon et al., 1996).

In the pupa the larva goes through total metamorphosis as mentioned before, but the development of the epidermis of the adult fly, including its appendages such as wings, eyes, legs and antennae has already begun in the embryo. Tissues called imaginal discs are set aside. These are sheets of epithelial cells, which proliferate, grow, and pattern during larval life. The body parts arising from the imaginal discs are excellent model systems for research in cell function and development. Morphological features of the wings and cuticle are products of important signalling

pathways and defects in these can be easily observed (MacDougall et al., 2004). Many signalling pathways active during development have been well characterised, and several, like EGFR, perform multiple functions in *D. melanogaster* development. In the imaginal discs, the EGFR functions as a factor for specifying cell fate and survival of postmitotic cells. Defective signalling consequently leads to a variety of patterning defects in the adult cuticle (Dominguez et al., 1998).

1.2.3 Genetic tools for investigating gene function

Mutations have occurred spontaneously in nature at all times in all species. In the beginning of the twentieth century, scientists used spontaneous mutations in the genome of *D. melanogaster* to study genetic inheritance. Since mutations that have scientific value occur very rarely by chance, scientists have, during the last twenty years developed methods to mutate the *Drosophila* genome with a higher frequency. This is most often done by the use of X-rays, gamma rays, or chemical mutagens such as ethylmethane sulfonate (EMS). Today, full saturation screens (mutating all genes in the flies genome) are done by EMS, which generate point mutations in the genome of the fly. The power of saturation screening in multicellular organisms was demonstrated by Christiane Nusslein-Volhard and Eric Wieschaus when they used this approach in an attempt to identify all important genes for embryonic pattern formation in one of the first large-scale *D. melanogaster* screens undertaken in the late seventies and early eighties (Nusslein-Volhard and Wieschaus, 1980).

Another way of introducing mutations in genes is by insertion of transposable elements. In all organisms, transposable elements are present as dynamic components of their genomes, thereby causing mutations and genetic variation. These elements are pieces of DNA requiring a host cell to replicate and proliferate. In *D. melanogaster* the most useful one is the P transposable element (P-element) (Miller et al., 1997). The P-element moves with high frequency in the *Drosophila* genome and is controlled by the availability of a transposase. In scientific experiments the P-element is controlled by a transposase not normally present in the fly. It has a major

advantage to other mutagenic agents by being easy to detect and map by sequencing of the neighbouring DNA. In most cases, the affected gene can be determined and the insertion mapped with minor efforts because the P-element preferentially inserts near the 5' end of the genes. A disadvantage of P-elements is their tendency to insert at specific genomic sites with a high frequency. This biased insertion makes it impossible to mutate all genes in the genome by simple P-element mutagenesis (Spradling et al., 1995). Almost any sequence can be inserted between the inverted repeats recognised by the transposase, and this makes the P-element extremely useful as a tool for other purposes, such as enhancer-traps, and more importantly, insertions of transgenes into the genome (Spradling et al., 1999).

UAS-GAL4 system

Model organisms have experienced a renaissance during the last decade, and the genetic toolbox has expanded enormously, particularly in *D. melanogaster*. The sequencing of the whole genome has allowed scientists to investigate almost all genes and processes. One of the most elegant tools for targeted gene expression is the GAL4/ UAS system.

GAL4 is a transcriptional activator identified in *S. cerevisiae* (Laughon et al., 1984; Laughon and Gesteland, 1984). The GAL4 protein regulates gene expression by binding to an enhancer called UAS (Upstream Activating Sequence) upstream of its target gene (Giniger et al., 1985). Expression of GAL4, not normally present in *Drosophila*, has no deleterious effect and can drive expression of a reporter gene under the control of a UAS sequence (Fischer et al., 1988). This technique has proven to be very powerful to express any gene in a temporal and spatial fashion *in vivo* (Brand and Perrimon, 1993). The gene of interest (the responder) is controlled by the presence of the UAS element, but the gene expression is silent when GAL4 (the driver) is absent. By keeping the UAS/ responder gene and GAL4 in separate fly lines, the responder gene expression is first achieved when the two fly lines are mated. GAL4 is not expressed ubiquitously, but is controlled by an enhancer in the *Drosophila* genome. Therefore GAL4 and the responder gene are only expressed in

tissues where the respective enhancer is activated. Since neither the GAL4 expression nor the silent responder gene is of any harm to the flies, when kept in separate fly lines, the responder gene can be either toxic, lethal, or have reduced viability when expressed. One example of such a responder gene is *reaper*. This gene can trigger programmed cell death. When a fly line carrying this silent reporter is mated to a fly line with a GAL4 driver, it causes cell death in those tissues where the driver is active and expresses GAL4 (Figure 4) (Greenspan, 1997).

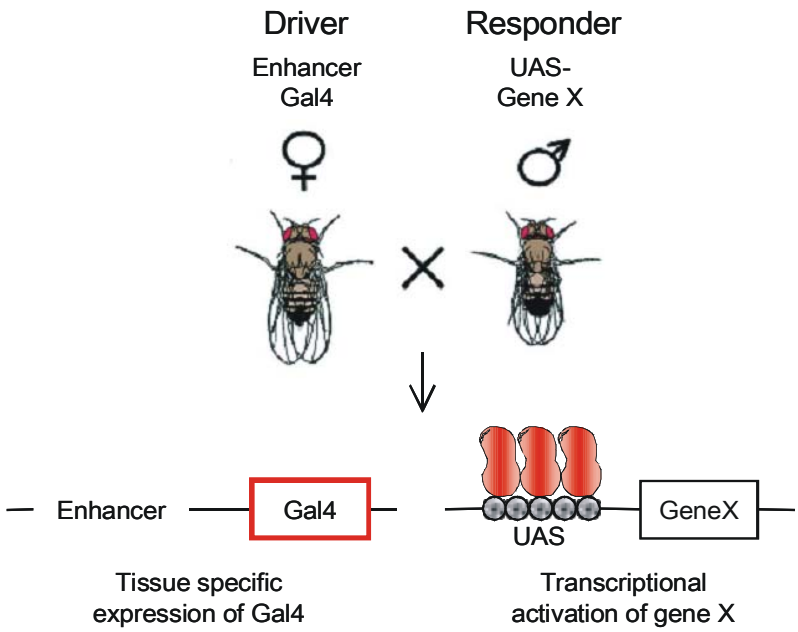

Figure 4 The GAL4/ UAS system. The two components from the driver and responder fly line are brought together in the offspring by a single cross. The expression of the Gal4 gene (red box) is driven by a time and tissue specific enhancer. The Gal4 protein (red) binds the UAS sequences (circles) and activates the expression of the gene of interest, Gene X (black box). (The figure is adapted from (St Johnston, 2002)).

Mosaic analysis

Some mutations cause early lethality in *D. melanogaster*. When studying processes late in development, a way of getting around this problem is the Flp/ FRT (Flip Recombinase Target) site-specific recombination system (Figure 5). This elegant method creates genetic mosaics at desired stages in development and overcomes the problem with early lethality by generating mutant groups of cells (clones) in an otherwise wild type environment. This enables scientists to address many biological questions. Originally, the genetic mosaics were generated by X-ray-induced mitotic

recombination, but the rate of recombination was very low. The cells in such a clone could be distinguished from the surrounding tissue only in very specific cases. The Flp recombinase is originally from yeast, but works very efficiently when expressed in *D. melanogaster* (Golic and Lindquist, 1989). By creating flies with transgenic FRT-sites at identical positions on homologous chromosomes, Flp-mediated recombination can be used to generate mitotic clones (Golic, 1991). If the site-specific recombination between the two homologues takes place after DNA replication in G2 phase of the cell cycle, the chromosome arm distal to the FRT-recombination site will be made homozygous. The daughter cells will inherit two copies of the region from one of the parental chromosomes, but this will only happen if the sister chromatids segregate appropriately. If the cells are heterozygous for a mutation and the mutation is located distal to the FRT-site, the recombination can be used to make a mutagenized chromosome arm homozygous in clones of cells. The homozygous mutant cells can be screened for a phenotype. A great advantage of this approach is that only the cells of interest are made homozygous by controlling where and when the recombination takes place. Regardless of their function in development, tissue specific mutant phenotypes in essential genes can be identified. Another advantage is that it is not necessary to go through two generations of flies, which is the case with traditional screens, to make the mutagenized chromosome homozygous. The F_1 generation, which is the first generation of progeny, can be screened for recessive loss-of-function phenotypes (St Johnston, 2002).

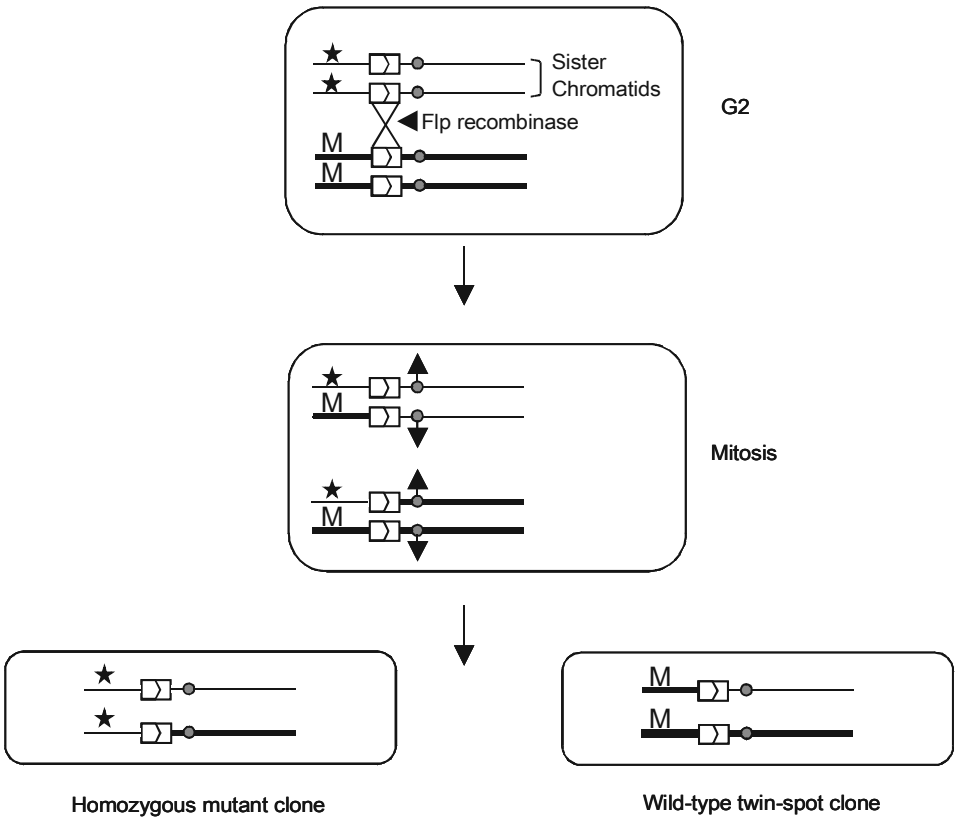

Figure 5 Using the FRT/ Flp system to generate clones in *D. melanogaster*. The upper picture shows a cell in the G2 phase of the cell cycle. The two homologue chromosomes, one indicated with thin lines and the other with thick lines, have replicated to form sister chromatides. On the upper sister chromatide pair an asterisks indicate a mutation. On the lower pair of sister chromatides the M indicates a marker upstream of the FRT-recombination site (indicated by the box). The Flp recombinase induces a flip between the chromosome arm distal to the FRT-recombination sites on the sister chromatide containing the mutation and the sister chromatide not containing the mutation**.** During mitosis the sister chromatides are segregated as indicated by the arrows and shown in the lower panel. The cell to the left has become heterozygous for the mutation and can be distinguished from the other cells by the lack of marker. The recombination has made one of the cells homozygous for the mutation and the cell can give rise to a clone of cells containing the mutation. The homozygous mutant cells can be screened for a phenotype. The cell arising from the chromatides with the marker, lacking the mutation, will behave as wild type. Adapted from (St Johnston, 2002).

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2. Manuscript

Characterisation of the *Fab1* phosphatidylinositol-3 phosphate 5-kinase in *Drosophila melanogaster*

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Abstract

Phosphoinositides play important roles in downregulation of growth factor receptors, such as the epidermal growth factor receptor (EGFR), through regulation of membrane transport and multivesicular body (MVB) biogenesis. Phosphatidylinositol-3-phosphate (PI(3)P) is required for the formation of intraluminal vesicles in MVBs and sorting of EGFR into these vesicles by recruiting PI(3)P binding effector proteins, such as Hrs. Another candidate effector of PI(3)P is the PI(3)P binding PI(3)P 5-kinase, *FAB1*, that synthesises one of the least studied phosphoinositides, PI(3,5)P_2, from PI(3)P. In yeast both Vps27 (Hrs) and Fab1 are necessary for correct membrane trafficking, and display morphological defects, such as enlarged vacuoles when mutated. We describe the isolation and characterisation of the *Drosophila melanogaster FAB1*

gene (termed *fab1*) and its mutants. The *fab1* gene encodes a protein of 1809 amino acids with similar domain architecture as in other eucaryotes. Immunolocalisation of the Fab1 protein shows colocalisation with Rab5, a marker for early endosomes (EEs), FYVE, a marker for EEs and MVBs and Rab7, a marker for MVBs. Analysis of fluid phase endocytosis revealed that *fab1* mutant cells are larger than wild type cells with accompanying enlarged endocytic vesicles, and a block in endosome to lysosome fusion. Initial analysis of EGFR signalling in *fab1* mutants did not reveal increased signalling activity. Further analysis is needed to investigate the potential role of Fab1 in receptor downregulation.

Introduction

Tight regulation of membrane trafficking is important to ensure proper temporal and spatial delivery of membrane-bound cargo. The endocytic pathway transports, among other proteins, transmembrane proteins from the plasma membrane to the EEs and MVBs (Lloyd et al., 2002). One type of transmembrane protein is the growth factor receptor, EGFR. EGFRs are downregulated by endocytosis via sorting into MVBs and subsequent degradation in the lysosomes upon fusion with the MVBs. Defective EGFR downregulation may cause uncontrolled cell proliferation and cancer. Phosphoinositides (PIs) are lipids known to regulate receptor downregulation by recruiting effector proteins necessary for the receptor sorting into the MVBs. Previously it has been established that phosphatidylinositol-3-phosphate (PI(3)P) regulates endocytic membrane trafficking by recruiting intracellular effectors containing a PI(3)P binding FYVE (conserved in *FAB1*, YOTB, Vac1, EEA1) domain, such as hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs). Hrs plays an important role in the sorting of monoubiquitinated EGFR into the inner vesicles of MVBs for degradation in the lysosomes (Raiborg et al., 2003). The Hrs-protein complex is believed to deliver its ubiquitinated proteins to ESCRT-I, a complex consisting of three subunits (Vps23, Vps28 and Vps37) by binding the subunit Vps23 in yeast and Tsg101 (Tumour susceptibility gene 101) in mammalian

cells, both containing ubiquitin binding domains (Bache et al., 2003; Katzmann et al., 2003; Lu et al., 2003; Pornillos et al., 2003). The ubiquitinated protein is transported, via a second protein complex ESCRT-II (Vps22, Vps25 and Vps36), into an intraluminal vesicle, which is formed by the polymerisation of a third multisubunit complex, the ESCRT–III (Vps2, Snf7, Vps20 and Vps24) on the endosomal membrane (Raiborg et al., 2003). *Saccharomyces cerevisiae* cells that lack one or more of the ESCRT-subunits do not form the intraluminal vesicles and have defective protein sorting to the vacuole (equivalent to the mammalian lysosome) (Katzmann et al., 2003). The yeast FYVE domain containing kinase, Fab1 (fragmented and binucleated 1), is also involved in membrane trafficking and receptor sorting into the MVBs. *FAB1* was first discovered in a screen for defects in protein sorting. The yeast cells had enlarged, deacidified vacuoles and the cell membrane receptor, Ste3, failed to be sorted into the vacuolar lumen, indicating that Fab1 enzymatic activity plays a crucial role in regulating membrane trafficking (Dove et al., 1997). Fab1 and the mammalian homologue PIKfyve are phosphatidylinositol-3-phosphate 5-kinases (PI3P-5Ks) synthesising PI(3,5)P_2 from PI(3)P (Sbrissa et al., 2002). Studies of PIKfyve at the protein level have revealed a widespread expression of the protein in various cells and tissues (Sbrissa et al., 2000). Analysis of the intracellular membrane fraction has suggested that PIKfyve associates with the membranes of the late endocytic pathway (Shisheva et al., 2001). Late endosomal PI(3)P is probably both a binding site for the FYVE-domain in Fab1/ PIKfyve and a source of PI(3,5)P_2 production (Sbrissa et al., 2002). In mammalian cells, overexpression of a dominant negative PIKfyve does not lead to elimination of the inner vesicles of MVBs, indicating that the PI3P-5K is not needed for the intraluminal vesicle formation. However, Fab1 might play an important role in the sorting of a subset of proteins into the intraluminal vesicles of MVBs (Reggiori and Pelham, 2002). The ESCRT-III complex contains a subunit, Vps24, that is able to bind PI(3,5)P_2, the catalytic product of Fab1. This finding suggests that PI(3,5)P_2 might function as an activator of ESCRT-mediated protein sorting (Whitley et al., 2003). In addition, PI(3,5)P_2 binding effector proteins might play a role in sorting of proteins into the MVBs. In

yeast, a protein, Ent3p, containing a PI(3,5)P_2 binding ENTH domain has been identified. Ent3p localises to the MVBs in a manner that is dependent on Fab1 kinase activity (Friant et al., 2003).

Thus, although numerous data suggest that Fab1/ PIKfyve may play a role in vesicle trafficking and protein sorting, the precise function of Fab1/ PIKfyve in these processes is unclear. To further investigate the role of Fab1, we analysed the effects of the loss of *fab1* in *Drosophila melanogaster* by studying three independent mutant fly lines encoding kinase domain deficient Fab1 proteins. Importantly, we demonstrate that loss of Fab1 might lead to impaired fusion between MVBs to lysosomes. The impaired endolysosomal fusion suggested a block or delay in endosomal membrane receptor degradation.

Results

Drosophila has one *FAB1* homologue

Drosophila fab1 like the yeast and mouse orthologs, *FAB1* and PIKfyve respectively, is believed to encode a phosphatidylinositol-3-phosphate 5-kinase (PI3P-5K). *fab1* is located on the R-arm of chromosome two and has been mapped to cytological band 54E9 (Figure 1C) (The FlyBase Consortium, 2003). Only one single *fab1* homologue was identified in the fly genome in FlyBase (The FlyBase Consortium, 2003) and there are no other genes predicted to be overlapping with *fab1*in the same region (Figure 1A). Three independent fly lines, $fab1^{8}$, $fab1^{21}$ and $fab1^{31}$, believed to be mutated in the *fab1* genetic region were isolated during an EMS mutagenesis screen for defects in *Drosophila* embryonic tracheal development. The mutants did not complement a deficiency mutant $fab1^{30w}$ lacking a region on chromosome two including the whole *fab1* gene (Figure 1B) (Mohr and Gelbart, 2002). The predicted structure of the *fab1* gene was confirmed by sequence analysis of a full length EST cDNA clone, GH01668 (2003). The clone predicted a open reading frame of 1809 amino acids (Figure 1D). Aligning this protein sequence against the sequences in *Saccharomyces cerevisiae*, *Caenorhabditis elegans* and *Mus musculus* (Appendix 1),

shows an overall sequence identity of 26,6 % (37,7 % similarity) and the protein harbours several evolutionarily conserved domains with functional significance (Shisheva, 2001) (Appendix 1). Database analysis with PFam (Bateman et al., 2004) of the sequence showed domains similar to a PI(3)P binding FYVE domain (amino acids 181-247) located at the N-terminus of the protein (Ponting and Bork, 1996), a chaperonin-like region (amino acids 424-824), found in proteins implicated in actin and tubulin folding, and a putative catalytic kinase domain (amino acids 1563-1796) found in phosphatidylinositol 5-kinases (PI5Ks) and phosphatidylinositol 4-kinases (PI4Ks) (the domains are indicated in Figure 1D and Appendix 1). The FYVE domains in *Drosophila* and mouse show 71,6% similarity and 62,7% identity and the kinase domains show 61,9% similarity and 50,4% sequence identity. The kinase domain of *Drosophila* Fab1 harbours two important sites, an ATP binding site and a lipid specificity site that are of functional importance and depicted in Figure 1D (Shisheva et al., 1999). In fact, *fab1* belongs to an evolutionary ancient gene family represented by a single-copy gene also identified in *Schisosaccharomyces pombe*, *Arabidopsis thaliana* and *Homo sapiens* (Shisheva, 2001).

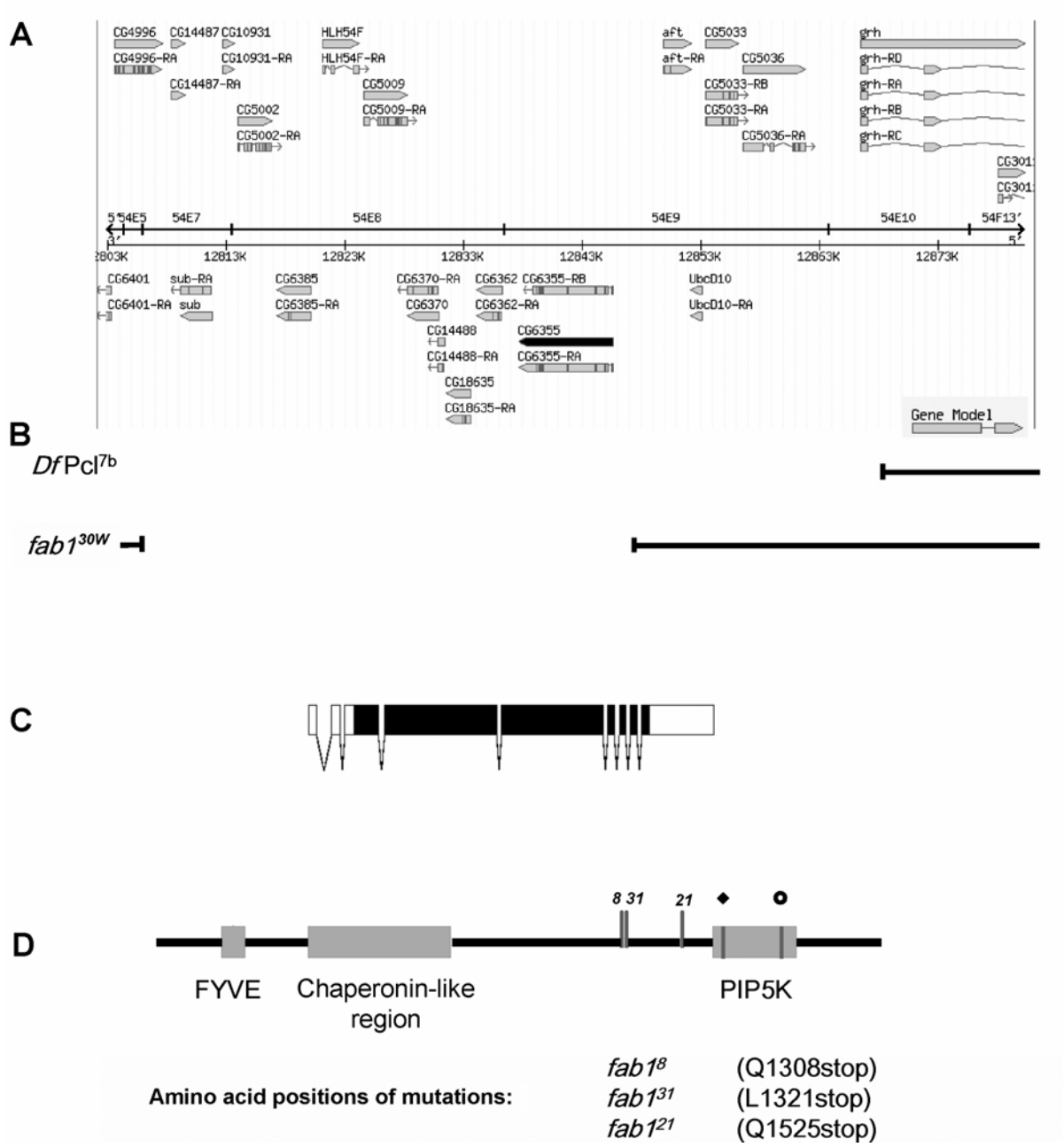


Figure1 *Drosophila fab1*: Genomic location, deficiencies, structure and mutational analysis. (A) *Drosophila fab1* CG6355, highlighted in black, is located on chromosome two, cytological band 54E9 (mRNA splice variants are located above and below the gene, long form CG6355-RA and short form CG6355-RB giving rise to identical proteins). The figure shows other genes located close to *fab1*, but here are no other genes predicted to overlap *fab1* (adapted from FlyBase (The FlyBase Consortium, 2003)). (B) The figure is related to (A) and the region between the bars shows the region that is missing in the $DfPcl^{7b}$ and $fab1^{w30}$ deficiencies. $DfPcl^{7b}$ is lacking a region larger than the figure in (A), only depicted by one bar. $fab1^{w30}$ is a smaller deficiency, depicted by two bars. (C) The structure of *fab1* with UTR regions indicated by white boxes and intron (spaces with cone)/ exon (black) boundaries. (D) The protein structure of Fab1 containing a PI(3)P binding FYVE domain, chaperonin-like region and PIP5K kinase domain. The diamond indicates the site for ATP binding and the circle indicates the lipid specificity site. The numbers show the location of the three *fab1* lesions, $fab1^{8}$, $fab1^{31}$ and $fab1^{21}$ respectively. The mutations are point mutations changing one amino acid to a stop codon leading to a truncated, kinase deficient protein. The exact amino acid position is shown at the bottom of (D): $fab1^{8}$ changes amino acid number 1308, a glutamine, to a stop codon, $fab1^{31}$ has a change in amino acid 1321, a leucine to a stop codon and $fab1^{21}$ has a change in amino acid number 1525, a glutamine to a stop codon.

Lethal phase of *Drosophila fab1* heteroallelic mutants

No animal with heteroallelic combinations of *fab1* mutations reached the adult stage and the *fab1* mutations identified are thus recessive lethal mutations. In order to check the lethal phase of *fab1* deficient animals, we observed the percentage of mutant larvae reaching the pupal stage. We generated homozygous and heterozygous *fab1* mutants by crossing flies with the mutations $fab1^{8}$, $fab1^{21}$ and $fab1^{31}$ balanced over a balancer chromosome *Cyo, Kr-GFP* to the deficiency $fab1^{30w}$ over the same balancer. Since the mutation is recessive, the heterozygous mutants will not be affected and express a wild type phenotype. Because the balancer chromosomes are lethal when homozygous, 25% of the larvae will die. Only 75% of the embryos can become larvae and therefore the expected percentage of mutant larvae will be 33% of the total number of larvae. We also analysed the lethal phase of *Drosophila* larvae lacking the region where *fab1* is positioned by crossing flies heterozygous for the $fab1^{30w}$ and $DfPcl^{7b}$ deficiencies (Figure 1B). The percentage of larvae actually surviving to the pupal stage was around 25%, instead of the expected 33% (Table 1). Some mutant larvae might actually die before they reach the pupal stage and thereby decrease the number of *fab1* mutant pupa. No *fab1* homozygous mutants were observed to hatch from the pupa and such mutants are therefore pupal lethal.

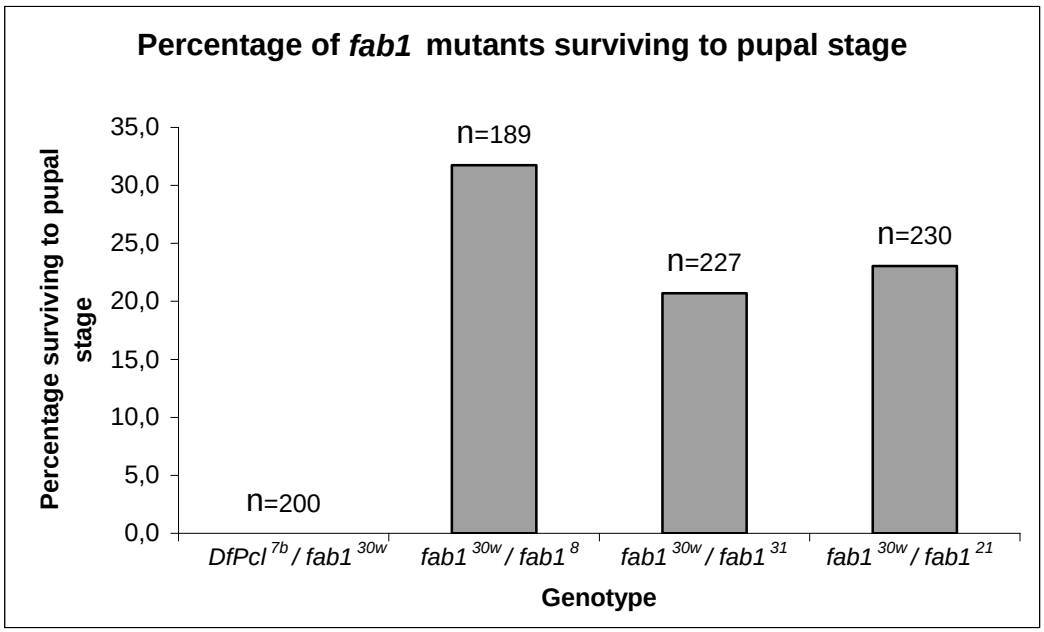


Table1. Approximate percentage of mutant larvae surviving to the pupal stage. This figure shows the approximate percentage of *fab1* mutant larvae that survive to the pupal stage. Twenty to 30 % of heteroallelic animals reached the pupal stage, while $DfPcl^{7b}$/ $fab1^{30W}$ die as late L3 larvae (not shown). Mutant flies hatching from the pupa have not been observed (n is total number of larvae counted).

Characterisation of the *fab1* mutants by sequencing

In order to find the mutations and map them to the *fab1* gene, we sequenced the genomic DNA isolated from *fab1* mutants. We used *fab1* heterozygous mutant flies from all three mutant lines, balanced over a GFP tagged chromosome, to perform a cross and generate homozygous *fab1* mutant larvae. All larvae lacking GFP expression were thus homozygous *fab1* alleles. The non-expressing GFP-larvae were selected and genomic DNA was isolated and sequenced. The sequencing showed nonsense mutations upstream of the kinase domain in all three mutant strains. In $fab1^{8}$ the mutation was a CAG-to-TAG nonsense mutation at amino acid number 1308 changing a glutamine to a stop codon (Q 1308 Stop), $fab1^{21}$ was a TTG-to-TAG nonsense mutation at amino acid number 1321 changing a leucine to a stop codon (L 1321 Stop) and $fab1^{31}$ was a CAG-to-TAG nonsense mutation at amino acid 1411 changing a glutamine to a stop codon (Q 1525 Stop) (Figure 1E). Based on their lethal phase, all three alleles are likely loss-of-function alleles.

Generation of an antibody against Fab1

Polyclonal antibodies were generated to the N-terminal half (amino acids 1-400, #267 anti-N-Fab1) and C-terminal half (amino acids 1423-1809, #269 anti-C-Fab1) of the Fab1 protein (the N-terminal antibody gave the best results and is the one used in all experiments). The protein encoding sequences were cloned into the pMAL-C2 vector making a MBP fusion and purified (Figure 2A). The fusion proteins were purified and sent for immunisation in rabbits. The resulting antiserum was affinity purified using the previously obtained MBP-fusion protein on an affi-gel (Figure 2B). The #267 anti-N-Fab1 was tested in L3 larvae on wild type Garland cells and Garland cells from animals lacking the region containing the *fab1* gene. The tissue staining analysis showed specific staining of vesicular structures in the wild type cells, but not in the *fab1* deficient cells where the entire *fab1* gene was missing, $fab1^{30w}$ / $DfPcl^{7b}$ (Figure 3A). Western blot analysis of larval extracts using the same antibody detected a weak band of 200 kD in wild type, Fab1 deficient and mutant animals. In the $fab1^{31}$ mutant a weak band of 150 kD was detected by the #267 Fab1-antibody in addition to

the 200 kD band, suggesting that a 1321 amino acid truncated protein is expressed in the mutant animal (Figure 3B). The presence of full-length protein in the L3 larvae of the Fab1deficient and the mutant animals suggests that maternally deposited Fab1 is stable and compensates for the loss of zygotic Fab1 during embryonic development. This supports the fact that the lethal phase of *fab1* mutants are in the pupal stage. The staining with #267 anti-Fab1 shows that the #267 anti-N-Fab1 is specific.

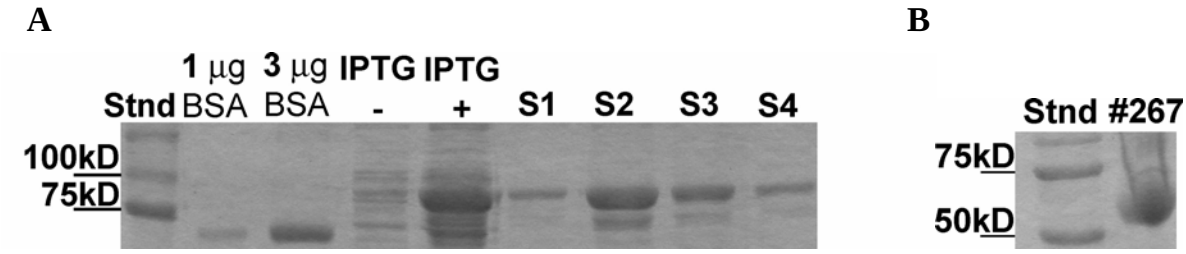
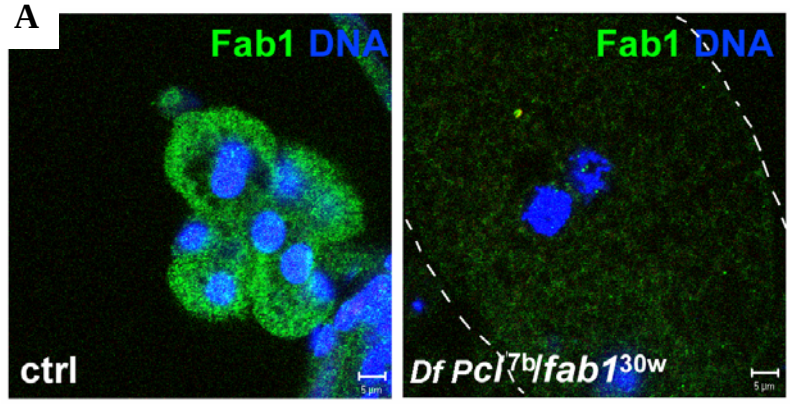


Figure 2. MBP fusion protein for immunisation and affinity purified protein gels. (A) The standard (Stnd) shows a band of 100 kD and a band of 75kD. In the IPTG- lane (lane 4) a band around 90 kD can be seen. In the IPTG+ lane (lane 5) a stronger band of the same size can be seen. These bands are the MBP-fusion proteins. The expression of the protein is increased with addition of IPTG. The lanes S1-4 show different MBP/ Fab1-N-fusion protein elutions, with the highest protein concentration in lane S2. 1µg and 3µg BSA in lane 2 and 3 are included to measure protein concentration in lane S1 to S4. (B) Gel picture showing the affinity purified #267 anti-Fab1 antibody heavy chain at around 50 kD.



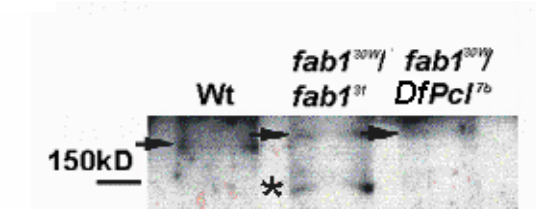


Figure 3. Antibody staining of wild type and *fab1* mutant Garland cells and Western blot analysis of wild type and *fab1* mutant larvae. (A) The confocal image shows #267 anti-Fab1-N antibody staining in green in wild type Garland cells (left) and a *fab1* deficient Garland cell (right). Nuclei are stained in blue Garland cells contain two nuclei in each cell. (This experiment was performed by Tor Erik Rusten.)

(B) Western blot analysis of third instar larvae (L3) showing a band of 200 kD in the wild-type, mutant and deficiency larvae indicated by arrows. A band of 150 kD can bee seen in the mutant, suggesting that a 1321 amino acid truncated form of Fab1 is expressed in the mutant (indicated by a asterisk).

Characterisation of the subcellular localisation of the Fab1 protein

To determine the subcellular localisation of Fab1, expression was examined in imaginal discs of third instar larvae (L3). The facts that PI(3)P is found on endosomal membranes and that Fab1 contains a PI(3)P binding FYVE-domain similar to that found in Hrs, made us ask the question whether Fab1 is to be found on the same structures as Hrs or on later endocytic structures. This was investigated using GFP fusion proteins of the endocytic markers Rab5, dbFYVE and Rab7. The Rab5-, dbFYVE- and Rab7-GFP fusions are located downstream of a UAS sequence in transgenes inserted in the *Drosophila* genome, and the Ptc-GAL4 driver expresses the GFP-fusion protein in a stripe in the imaginal discs in the L3 larvae (Figure 4A). Rab5 labels EEs, dbFYVE labels EEs and MVBs and Rab7 labels MVBs (Figure 4B). The discs were stained with #267 anti-N-Fab1 and showed staining of vesicular structures that colocalised with all three endocytic markers, supporting the hypothesis that Fab1, like Hrs, is an important part of the endocytic pathway (Figure 4C, D and E).

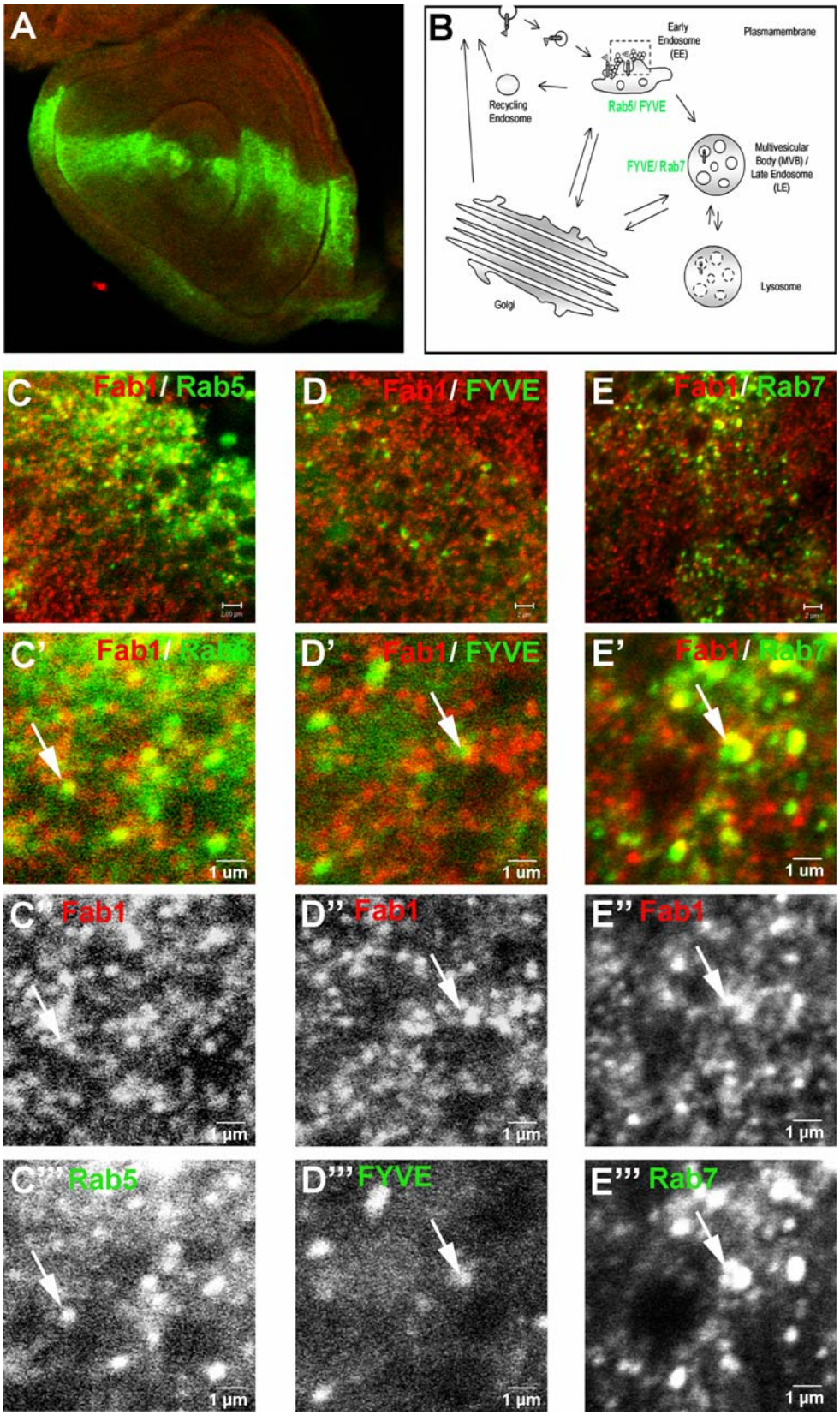


Figure 4. Colocalisation of Fab1 with endosomal markers. (A) The imaginal disc in a third-instar larva expressing GFP-Rab5 fusion protein in a stripe (GFP–dbFYVE and -Rab7 are expressed in a similar manner). (B) A schematic presentation of the endocytic pathway in a cell showing the location of Rab5 on EEs, dbFYVE on EEs and MVBs and Rab7 on MVBs. (C, D and E) The panels are showing enlarged parts of the imaginal discs expressing Rab5, dbFYVE and Rab7 respectively in a stripe (green structures) and staining with #267 Fab1-N antibody (red structures). (C'-C''') Rab5-GFP colocalises with #267 Fab1-N antibody in distinct 0,5 µm-sized structures (arrow indicate one of the vesicles that show overlap). (D'-D''') dbFYVE-GFP colocalises with #267 Fab1-N antibody in distinct 0,5 µm-sized structures (arrow indicate one of the vesicles that show overlap). (E'-E''') Rab7-GFP colocalises with #267 Fab1-N antibody in distinct 0,5 µm-sized structures (arrow indicate one of the vesicles that show overlap).

Failure in endosome-to-lysosome fusion in *fab1* mutant Garland cells

To determine if Fab1 functions in endocytic vesicle trafficking, the internalisation of fluid phase tracers was investigated in third-instar larval Garland cells, large cells with a high rate of fluid phase endocytosis (Narita et al., 1989). Wild type Garland cells showed strong labelling of peripheral vesicles after 5 min incubation with Oregon green dextrane (OGD), indicating that the dye internalises rapidly into endosomes. Mutant cells were much larger than wild type cells but showed strong labelling of peripheral vesicles, suggesting that dye internalisation was not significantly impaired. The endosomes did not colocalise with the lysosomal marker (lysotracker) after 5 min uptake and 0 min chase (Figure 5 A and C). After 40 min chase, almost all the OGD in wild type cells colocalised with lysotracker, whereas in the *fab1* mutant cells the marker still seemed to be trapped in endosome-like structures. However, many labelled endosomes in the mutant cells were much larger than those observed in the wild type cells (Figure 5 B and D). These results indicate that internalisation is not affected by the *fab1* mutation, but the fusion between endosomes and lysosomes is defective or delayed in the f*ab1* mutant cells.

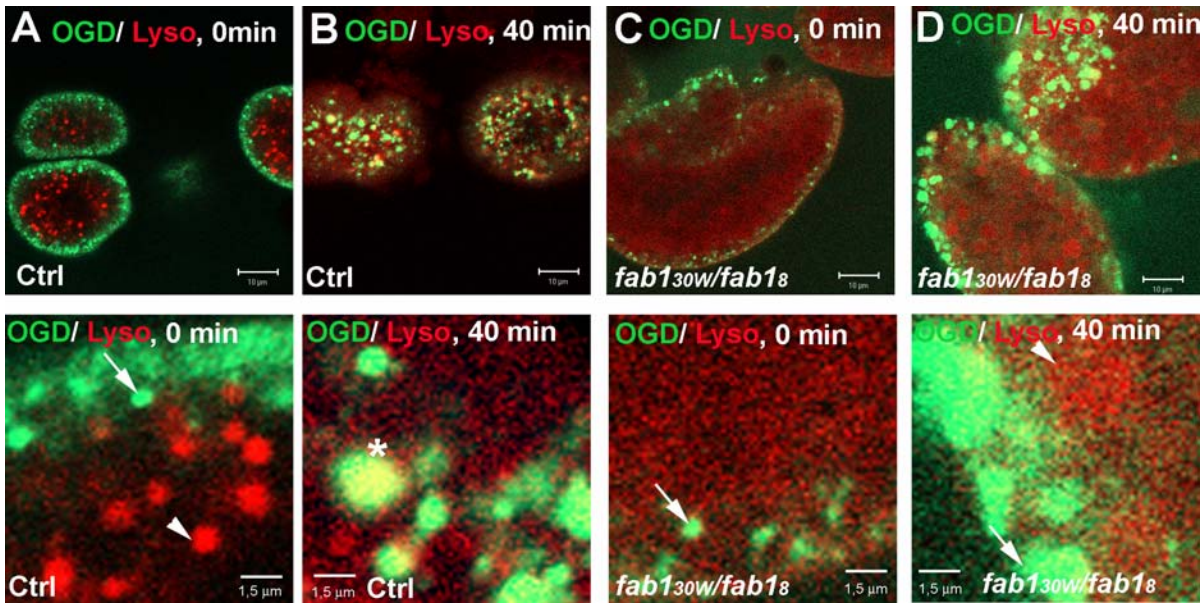


Figure 5. Failure in endosome to lysosome fusion in *fab1* mutant Garland cells. The lower panels (scale bars 1,5µm) are enlargements of the upper panels (scale bars 10 µm) (A) Wild type cells take up the Oregon green dextran dye (OGD) in green (arrow) and show peripheral labelling after 5 min uptake, but no colocalisation with the red lysosomal marker lysotracker (arrowhead). (B) After 40 min chase the OGD is colocalising with lysotracker (asterisk), indicating endosome to lysosome fusion. (C) Uptake of OGD in a *fab1* mutant cell after 5 min and almost no lysosomal labelling indicating that lysosomes are less acidic. (D) After 40 minutes chase the OGD can still be seen in the periphery. The endosomes are larger than wild type and no colocalisation can be seen between the weak lysosomal staining and OGD.

Characterisation of EGFR downregulation in *fab1* mutant embryos

To investigate the hypothesis that *fab1* mutants had impaired MVB sorting, we examined the downregulation of the EGFR. This receptor is downregulated by endocytic internalisation, sorting into the MVBs and subsequent degradation upon fusion with the lysosome. If the sorting into the MVBs is defective, the EGFR will remain in the limiting membrane and therefore give rise to increased signalling, as observed in *hrs* mutants (Lloyd et al., 2002). In the *Drosophila* embryo, a precise pattern of sensory organ precursor cells in the peripheral nervous system (PNS) provides a useful model to study EGFR signalling. The *Drosophila* PNS comprises approximately 600 neurons and 1200 associated cells organised in a segment specific pattern. In the abdominal hemi-segments (A1-A7) the neurons are organised in three clusters along the dorsoventral axis. The PNS sensory organ is divided in two parts, internal and external. The internal part is called chordotonal organs (Chs) and at the lateral (L) position five clustered cells, LCh5, are located. EGFR signalling regulates the cell number in LCh5. Two of the five chordotonal organs in LCh5 are determined by EGFR signalling. Overactive signalling will give rise to more than two neurons such that the total number of neurons would be more than five. Absence of EGFR signalling will yield only the three neurons not determined by EGFR signalling (Figure 6B) (Okabe and Okano, 1997; Rusten et al., 2001). We investigated the possible EGFR overactivation by staining mutant embryos with a marker that labels the neurons of the PNS, including the LCh5 (Figure 6A). By confocal microscopy we could clearly see five chordotonal organs in wild type embryos (Figure 6D). We could not observe any effect of overactive EGFR signalling as expected from the previous observed results of what seemed to be defective endosome to lysosome fusion in the Garland cells (Figure 6 E and F).

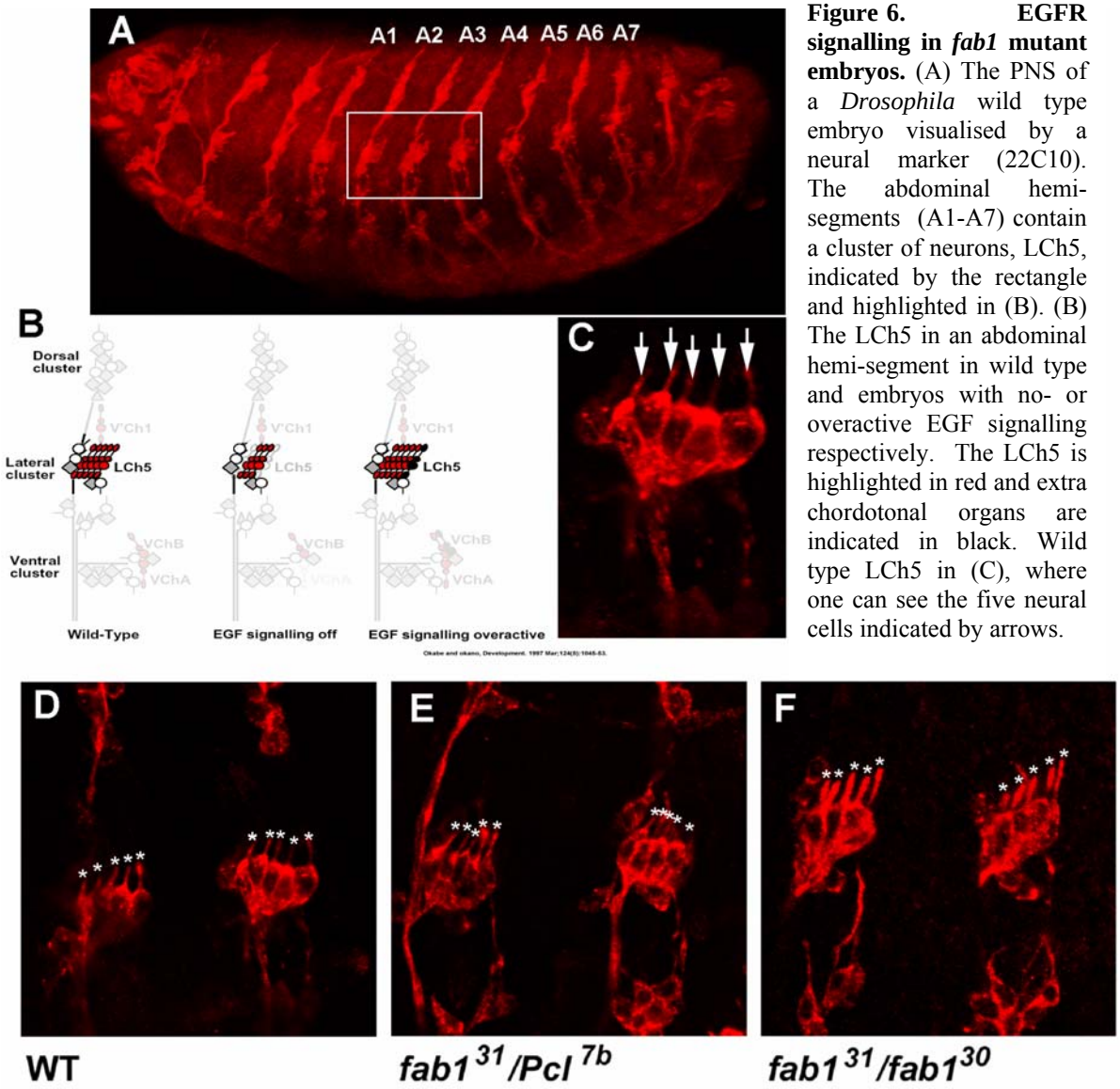


Figure 6. EGFR signalling in *fab1* mutant embryos. (A) The PNS of a *Drosophila* wild type embryo visualised by a neural marker (22C10). The abdominal hemi-segments (A1-A7) contain a cluster of neurons, LCh5, indicated by the rectangle and highlighted in (B). (B) The LCh5 in an abdominal hemi-segment in wild type and embryos with no- or overactive EGF signalling respectively. The LCh5 is highlighted in red and extra chordotonal organs are indicated in black. Wild type LCh5 in (C), where one can see the five neural cells indicated by arrows. (D) Wild type embryo showing two LCh5 organs with five neurons each indicated by asterisks. (E) and (F) *fab1* mutant embryos showing two LCh5 organs with five neurons each (indicated by asterisks). The mutant embryos show no signs of overactive EGF signalling by increased number of neural cells as predicted in (B). Figure (B) is adapted from (Okabe and Okano, 1997).

Discussion

The yeast phosphatidylinositol kinase, Fab1, has been implicated in membrane trafficking and receptor sorting. We have identified and characterised the *Drosophila* Fab1 ortholog, which is highly conserved between flies, yeast and mammals. Characterisation of three *fab1* mutant alleles revealed point mutations predicting truncated proteins, which all lack the C-terminal part of the protein, containing the kinase domain. These alleles genetically behave as null mutations. Immunolocalisation of endogenous Fab1 showed that the enzyme is localised to the endocytic pathway, and similar to previous results from yeast, *Drosophila fab1* mutants display reduced lysosomal acidity. In contrast to earlier findings in yeast, analysis of fluid-phase endocytosis has revealed enlarged endosomes rather than lysosomes (equivalent to the yeast vacuoles) and a block or delay in the endosome-to-lysosome transport. This suggests a block or delay in lysosomal degradation of transmembrane receptors.

Roles of Fab1 in endocytic trafficking

Yeast cells containing a point mutation in the lipid kinase domain of *FAB1* have reduced PI(3,5)P_2 production, altered vacuole morphology, reduced acidity and endocytic protein sorting defects. Lysotracker staining of *Drosophila fab1* mutant cells is severely reduced compared to wild-type cells. The reduction of acidity in the lysosomes in the *fab1* mutant cells could be due to the mislocalisation of lysosomal proton pumps (Shaw et al., 2003). To check that the lysotracker stained structures are lysosomes, it would be useful to create an antibody against LAMP. Moreover, endosome to lysosome fusion is severely reduced or blocked. This impaired fusion between endosomes and lysosomes has been confirmed by electron microscopy (EM) studies (Andreas Brech, data not shown). Fluid-phase tracer studies of *fab1* mutant Garland cells have revealed a dramatic enlargement of cell size and endocytic structures. Very similar phenotypes have been found in *Drosophila* cells lacking Hrs. In *hrs* mutants endosome-to-lysosome fusion appears normal, but it is suggested that

an inability to invaginate the endosomal membrane causes the enlarged endosomes (Lloyd et al., 2002). In the *fab1* mutants the cause of the increased sizes of the cells and endocytic structures are unknown, but it could be due to a $PI(3,5)P_2$ effector protein important for membrane invagination. In yeast, one such effector, Ent3p, containing a $PI(3,5)P_2$ binding ENTH domain has been identified. This protein is involved in sorting of cargo into the MVBs. The actual sorting mechanism is unknown, but Ent3p is suggested to function similarly to epsin. The ENTH domain of epsin, once bound to its phosphoinositide, penetrates the membrane and inserts into one leaflet of the plasma membrane, thereby pushing aside surrounding lipids and inducing membrane curvature. Ent3p localises to the MVBs in a manner that is dependent upon Fab1 kinase activity and the $PI(3,5)P_2$ binding ability of the ENTH domain. Downstream effectors of Ent3p still remain to be identified (Friant et al., 2003).

A potential role for Fab1 in regulating cell signalling

Internalisation of cell surface receptors and their subsequent degradation in the lysosome is a mechanism thought to mediate signal downregulation. There is evidence suggesting that endocytic membrane trafficking regulates both the intensity of signalling and the colocalization of activated receptors with downstream signalling molecules (Ceresa and Schmid, 2000). Activated tyrosine kinase receptors (TKRs) are one example of receptors that are sorted into the lumen of the MVBs by interaction with Hrs and other proteins (Felder et al., 1990). When the TKRs are inside the MVBs they are unable to signal to downstream components (Lloyd et al., 2002). In yeast *FAB1* mutant cells, the transmembrane receptor Ste3 is not efficiently sorted into the vacuolar lumen, but instead localises to the limiting membrane of the vacuole (Shaw et al., 2003). Since we observed a severely reduced endolysosomal transport in the *fab1* mutants, it is likely that Fab1 is needed for degradation of transmembrane receptors as in yeast. Therefore, we investigated a potential increase of signalling of the TKR, EGFR, in the PNS of *Drosophila fab1* mutant embryos. This preliminary analysis did not reveal any detectable increase of EGFR signalling

in the absence of *fab1*. This could be due to the fact that EGFRs are properly sorted into the lumen of MVBs and signalling is therefore inhibited in a normal manner. Results from EM analysis have shown intraluminal vesicles in MVBs of *fab1* mutant larvae (Andreas Brech, data not shown). Another option is that the EGFR is dependent on Fab1 to be sorted into the lumen of MVBs (Shaw et al., 2003), and the reason for the undetectable overactivation of EGFR might be due to a well-characterised negative feedback mechanism, whereby transcription of EGFR is inhibited by hyperactivation of the receptor (Sturtevant et al., 1994). Yet another option is that maternally supplied *fab1* mRNA substitutes the zygotic loss, and it is therefore not possible to see an effect of the lack of Fab1 on the early stages of embryogenesis. These possibilities need to be studied in more detail and further analysis is needed to fully understand the role of Fab1in endocytic trafficking. Evidence suggests that both $PI(3,5)P_2$, synthesised by Fab1, and ubiquitin have a role in cargo selection and MVB sorting. Hrs, ubiquitin and the ESCRT complexes are involved in sorting of ubiquitinated cargo into the MVBs, while the role of $PI(3,5)P_2$ in multicellular animals is poorly understood (Raiborg et al., 2003). It is suggested that a subunit of the ESCRT-III complex, Vps24, is interacts with $PI(3,5)P_2$ in the MVB sorting process (Whitley et al., 2003). The yeast transmembrane receptor Ste3, like the mammalian EGFR, is ubiquitinated and sorted into the MVB. *FAB1* mutant yeast cells show mislocalisation of the majority of the Ste3 receptors to the vacuolar outer membrane in the absence of $PI(3,5)P_2$, but the Ste3 receptor does not show strict dependence on the lipid in the sorting process. In the absence of $PI(3,5)P_2$, detectable levels of Ste3 are to be found in the lumen of the vacuole. This finding is analogous to the localisation of a mutant Ste3 receptor, which lacks a lysine residue crucial for ubiquitination and sorting into the MVBs. Inefficient sorting of receptors into the MVB, could be due to the presence of multiple sorting signals on plasma membrane receptors, involved in recycling receptors from the endosomes, back to the plasma membrane. A sorting determinant such as $PI(3,5)P_2$ could play a role here as well as in sorting of cargo into the lumen of the MVBs (Shaw et al., 2003).

Data that could shed more light on the role of Fab1 could be the identification of a *Drosophila* Ent3p homologue and other proteins containing $PI(3,5)P_2$ binding domains. It will be interesting to check whether the EGFR in *Drosophila* is mislocalised in a similar manner to the Ste3 receptor in yeast. Further investigation is needed in order to determine if the absence of EGFR overactivation is due to sorting of EGFR into the MVBs or transcriptional suppression. To determine the localisation of $PI(3,5)P_2$, the ENTH domain of Ent3p could be used to generate a probe which binds the lipid. By creating a transgenic fly line carrying a GFP-ENTH fusion it would be possible to visualise $PI(3,5)P_2$ in a similar manner to PI(3)P, where the dbFYVE probe is used. By using the Flp/FRT system to induce clones it is possible to investigate the role of Fab1 in vesicle trafficking, receptor sorting and cell signalling when the maternal mRNA contribution is no longer a problem. In this study we have identified a previously unknown function of Fab1 in endosomal transport. This underlines the importance of studying vesicle trafficking in multicellular model organisms. It will be interesting to investigate other differences or additional roles that may exist between yeast and *Drosophila* in the control of vesicle transport.

Materials and methods

Materials

Chemicals

Advantage Taq polymerase New England Biolabs (MA, USA).
Advantage^{TM}2 Taq polymerase BD Biosciences Clontech (CA, USA).
Affi-gel Bio-Rad Laboratories (CA, USA)
Agar Life Technologies (Paisley, Scotland).
Ampicillin (50 mg/ ml) Bristol-Myers Squibb (New York, USA).
Amylose recin New England Biolabs (MA, USA).
Apple juice Tine Meierier (Ås, Norway).
Bromphenol blue Sigma Aldrich (MO 63178, USA)
BSA (bovine serum albumine) Sigma Aldrich (MO 63178, USA)
Buffers New England Biolabs (MA, USA).
Chloramphenicol (34 mg/ ml) Sigma Aldrich (MO 63178, USA)
Chloroform MERCK (Darmstadt, Germany)
dNTPs Roche Diagnistics GmbH (Mannheim, Germany).
DTT (dithiothreitol) Sigma Aldrich (MO 63178, USA)
Dye reagent concentrate Bio-Rad Laboratories (CA, USA).
EcoRI New England Biolabs (MA, USA).
EST *fab1* cDNA clone GH01668 with vector p-Mal-C2 and primers Invitrogen life Technologies (Maryland, USA).
Fly food: agar, molasses, syrup, yeast A/S Pals (Billingstad, Norway).
Formaldehyde Polysciences, Inc. (Pennsylvania, USA).
Glycerol MERCK (Darmstadt, Germany)
Glycine MERCK (Darmstadt, Germany)
GNS (goat normal serum) Sigma Aldrich (MO 63178, USA)
HCl MERCK (Darmstadt, Germany)
Hepes Sigma Aldrich (MO 63178, USA)
Heptane MERCK (Darmstadt, Germany).
IPTG (isopropylthio-β-D-galactoside) Saveen Werner AB (Malmö, Sweden).
KAc Sigma Aldrich (MO 63178, USA)
KCl MERCK (Darmstadt, Germany)
KH_2PO_4 MERCK (Darmstadt, Germany)
Lysotracker (1mM) in DMSO Molecular Probes (Oregon, USA).
Maltose Sigma Aldrich (MO 63178, USA)
Methanol VWR International (Oslo, Norway).
$MgCl_2$ MERCK (Darmstadt, Germany)
NaAc MERCK (Darmstadt, Germany)
NaCl MERCK (Darmstadt, Germany)
NaH_2PO_4 MERCK (Darmstadt, Germany)
Nipagin Sigma Aldrich (MO 63178, USA)
n-propylgallate Sigma Aldrich (MO 63178, USA)
Oregon green dextran (1mg/ ml) Molecular Probes (Oregon, USA).
Phenol Sigma Aldrich (MO 63178, USA)
Polyacrylamide 40% Bio-Rad Laboratories (CA, USA)
Ponceau S solution SERVA FEINBIOCHEMICA GmbH & Co KG (Heidelberg, Germany).
Propionic acid MERCK (Darmstadt, Germany)
Protease inhibitor Roche Diagnistics GmbH (Mannheim, Germany).
Protein^{TM} Sandards Dual color Bio-Rad, (CA, USA)
Proteinase K Boehringer-Mannheim GmbH (W. Germany, Germany).
SalI New England Biolabs (MA, USA).
Schneider medium Sigma Aldrich (MO 63178, USA)
SDS Bio-Rad Laboratories (CA, USA)
Sodium hypochlorite Lilleborg (Oslo, Norway).
Sucrose MERCK (Darmstadt, Germany)
Super signal® West ECL reagents Pierce qb Perbio (IL, USA).
T4-ligase New England Biolabs (MA, USA).
Trichloroacetic acid MERCK (Darmstadt, Germany)
Tris Sigma Aldrich (MO 63178, USA)
Triton X-100 Sigma Aldrich (MO 63178, USA)
$ZnCl_2$ MERCK (Darmstadt, Germany)

Antibodies

Primary antibodies: 22C10 mouse monoclonal antibody was obtained from the Developmental Studies Hybridoma Bank at the University of Iowa (diluted 1:20) (Fujita et al., 1982), rabbit polyclonal anti β-Galactosidase (diluted 1:4000) from Cappel (MD, USA), #267 rabbit polyclonal anti *Drosophila* fab1 N-terminal protein (diluted 1:1000 for western blot and 1:200 for tissue staining, this study). *Secondary antibodies*: Cy2- and Cy3-fluorocrome conjugated donkey anti rabbit IgG (diluted 1:200 and 1:500 respectively) and Cy3 fluorocrome conjugated goat IgG anti mouse (diluted 1:500) were all purchased from Jackson Immuno Research laboratories (1330 Fornebu, Norway).

Constructs

pMAL-C2

This vector is similar to pMAL-p2 except for a deletion of the *malE* signal sequence (bases 1531-1605). The vector is described in: http://seq.yeastgenome.org/vectordb/vector_descrip/PMALC2.html and supplied by New England Biolabs (MA, USA).

pOT2

The vector is described in: http://www.fruitfly.org/EST/pOT2vector.html and supplied by ResGen™ Invitrogen Corporation (CA, USA).

Drosophila stocks and genetics

Genetics

Flies were grown in vials (Regina industries LTD, Newcastle, England) on standard corn meal molasses agar (corn meal, agar, molasses, syrup, yeast, nipagin, propionic acid) at 18ºC or at 25ºC. In a standard fly cross each virgin female were crossed to a male in a 3:1 (female: male) ratio. Usually between 5 and 10 females were used. All crosses described were done at 25ºC.

Drosophila stocks

Df(2R)*14H10W-30*: a small deficiency uncovering the *fab1* locus (Mohr and Gelbart, 2002). In the text referred to as *fab1*^{30w}.

Df(2R)*Pcl*^{7B}: a large deficiency uncovering the *fab1* locus (http://rail.bio.indiana.edu/.bin/fbidq0.html?FBstBL-3064). In the text referred to as *DfPcl*^{7B}. Uncovered region: 054E08-F01;055B09-C01

fab1$^{SJB\ 8}$: amorph, nonsense mutation Q1308stop (this study). In the text referred to as *fab1*^{8}.

fab1$^{SJB\ 21}$: amorph, nonsense mutation Q1525stop (this study). In the text referred to as *fab1*^{21}.

fab1$^{SJB\ 31}$: amorph, nonsense mutation L1321stop (this study). In the text referred to as *fab1*^{31}.

Generation of homozygous mutant embryos

Homozygous *fab1* mutant embryos were generated by crossing: *fab1*^{31}/ CyO, f*tz*-LZ with *DfPcl*^{7b}/ CyO, *ftz*-LZ and fab1^{30w} / CyO, *ftz*-LZ. Mutant embryos were distinguished by lack of *ftz*-LZ expression detected by an antibody against β-Galactosidase.

Generation of homozygous mutant larvae

Homozygous mutant *fab1* larvae were generated by crossing: *fab1*^{8}/ CyO, Kr-GFP with *fab1*^{8}/ CyO, Kr-GFP, *fab1*^{21}/ CyO, Kr-GFP with *fab1*^{21}/ CyO, Kr-GFP and *fab1*^{31}/ CyO, Kr-GFP with *fab1*^{31}/ CyO, Kr-GFP. Homozygous *fab1* animals were distinguished by the lack of Kr-GFP expression.

Methods

Molecular biology

PCR

DNA was amplified using Advantage™2 Taq polymerase using standard procedures. The following conditions were used for a 50 µl reaction: 50 ng template DNA, 5 µl 10x reaction buffer, 1µl dNTPs (stock: 10 mM), 1 µl of each primer (stock: 1µg/µl), 1 µl Polymerase, H_2O to 50 µl. Denaturation: 30 seconds at 94°C, annealing: 30 seconds mostly at 60°C (temperature depending on primers), elongation: 1-3 min depending on length of DNA at 72°C, 30 cycles, primers were ordered from Invitrogen, life technologies (CA, USA).

Restriction digests, gel electrophoresis, ligations and transformations

Restriction enzymes, T4-ligase and corresponding buffers were used as recommended by New England Biolabs. Digests, ligations and agarose gel electrophoresis were carried out using standard techniques (Sambrook, 2001). Ligation reaction or purified plasmids were transformed in $CaCl_2$ competent DH10α or BL21 *E. coli* cells using standard procedures heat shock transformations (Sambrook, 2001). Bacteria were plated out on Luria Broth (LB)-agar plates, standard protocol (Sambrook, 2001) containing ampicillin for selection.

DNA purification

DNA was purified from E. coli using the QIAquick® PCR purification kit protocol (Quiagen, Hilden, Germany) or for mini preps the Wizard® Plus SV Minipreps DNA Purification System (Promega, WI, USA), following the protocols described in the handbook provided by the supplier. DNA fragments separated on agarose gels were purified using the QIAquick® Gel Extraction kit (Quiagen, Hilden, Germany) following the protocols described in the books provided by the suppliers. For mini

preps, bacteria were grown over night (ON) at 37 °C in LB with ampicillin (100µg/ ml).

Isolation of genomic DNA and mutational analysis

For each *fab1* mutant strain, 30-50 homozygous *fab1* mutant larvae were collected and homogenised in an Eppendorf tube in 0.5 ml DNA extraction buffer (0.1 M NaCl, 0.2 M sucrose, 0.1 M Tris-HCl, pH 9.1, 0.05 M EDTA, 0.5 % SDS, distilled water to 100 ml) in a 65 °C water bath. The larvae were incubated for at 65°C for 30 min and 75 µl 8 M Kac was added. The sample was incubated 30 min on ice, centrifuged for 1 min, max speed (20,800 x g) in a tabletop centrifuge (Eppendorf centrifuge 5417c equipped with a F45-30-11 rotor, BB Lab as, Skårer, Norway). The supernatant was transferred to a fresh tube. This procedure was repeated once. Ethanol was added to a final concentration of 70% and the sample was left for 5 min at room temperature (RT). The sample was then centrifuged at max speed (20,800 x g) for 5 min at RT (Eppendorf centrifuge 5417c, as used before), after which the supernatant was removed and the DNA washed in 70% ethanol. The supernatant was removed, washed and centrifuged once more and the sample air-dried for 5 min. 200 µl TE (100 mM tris pH 7.4, 10 mM EDTA pH 8), pH 8, was added and the DNA pellet was dissolved at 65 °C for 30 min by vortexing and pipetting. The sample was centrifuged at max speed (20,800 x g) for 5 min (Eppendorf centrifuge 5417c, as used before) and the pellet was discarded. 20 µl 5 M NaCl, 4 µl 0.5 M EDTA, 0.6 µl 10 mg/ml RNAseA (final conc. 20ug /ml) was added and the sample was incubated at 37 °C for 30 min 2.1 µl 10mg/ ml Proteinase K was added and the sample incubated at RT for 30 min with the addition of 1 volume Phenol/Chloroform to the aqueous solution. The sample was vortexed ~30 sek to form an emulsion and centrifuged 1-2 min (20,800 x g) (Eppendorf centrifuge 5417c, as used before) until the phases separated. The aqueous phase was retained. This procedure was repeated with Phenol/ Chloroform, until no protein was left at the interface. 1/10th volume 3 M NaAc ph 5.2 and 2 volumes ethanol (70% total in solution) were added, mixed and DNA precipitated at -20 °C for 30 min. The sample was centrifuged for 5 min at max

speed (20,800 x g) at RT using a tabletop centrifuge (Eppendorf centrifuge 5417c, as used before), the pellet was washed with 70% ethanol. DNA was dried and finally dissolved in 50 µl TE, pH8.

On the basis of the published genomic sequence of the *fab1* gene region, 21 different primer pairs were designed to amplify the whole *fab1* gene region from the three *fab1* mutant strains by PCR (2003).

5'-3' Sense	Antisense
TTTTGTATGTCCCAGTTGCATTG	CAAATGAAATTCATGAAGCTGTGG
CTGCTGTCCGAACCAAATG	AGCTGCATCGGCAAGATATC
GGCAAAAAAAAACAGCTGATATG	GTAGTAATCGAAAGTGCCAATCG
CTCACCATTAAGCTAATTGCC	CGACAGTGCTTAAATATTGGAG
CTGTGAGTGTGGCAAAATTATC	GTCGGGGTGTTAGGAGTTG
CTAACCAAGGACCGTGAGTC	GAAACGGTGCGCCCCTG
CCAATAGTGAGACACGTGGCAC	CTGCCACTATCTTGTACTTCCAG
TGGCGATCTCAAAGTGTGCAAC	GAAACCAGCGGCTAGCATAG
GTCAGCGTCTGATTGAGTTTC	ACAATCTTCGAGTCCTTGCG
AAGCCAGAGCATTGTAGTAACG	CCTCTGAGGAGACAGGTG
CAAGCTGGGCTATTGCAATG	AAATAAGGTAGGGGAAATGTCAG
TAGCTGTGGAACCGCGTTAC	AGACGTTGATGGTTCTGCGG
AAAAAAGAACGCCGAAGTGATC	CGATGCAATGAGTGTTCACATAC
AGATGCGCTTCCATGGCC	CCAGAGTCGATGTGATGCG
CACGGATAACAGGGCTACTG	CCGCTAATGGAATTTTTCGC
TCTCAGTTCAGTGATTGCATAC	TCCTCCTCCTGATTGGAATC
GCACCGGGTCCGAAATG	CCTCTCTGAGCCCTTAAGATC
ACTTACGCATTTTTAATACTAGTTC	GATTGACCACGGTCGGATC
TACATTCGAACCTTTACGCTGG	GAAACCATGCTTATGAGACTCC
GAAATTATTATCATGATGGAGACC	TACAACTTGACCGCTCAGATC
ATGTCGGAAAGTTGGCGTGC	CATGTTAGTTACCCTCC

These primer pairs gave approximately 500 bp-sized overlapping fragments spanning the entire *fab1* genomic region. The PCR reactions were resolved on agarose gel and extracted as previously described. The purified DNA was sent for sequencing by AGOWA (Berlin, Germany) and aligned against genomic DNA CG6355 (2003) and one other by using software Vector NTI, AlignX 2003 provided by InforMax Inc. (Maryland, USA) to find the mutations.

cDNA library sequencing

DH10α *E. coli* cells were transformed with pOT2-GH01668 EST *fab1* cDNA clone and selected on chloramphenicol (20 μg/ml) agar plates. The DNA was purified by Wizard® Plus SV Minipreps DNA Purification System and sent for sequencing to AGOWA (Berlin, Germany). The same primers were used as for the genomic DNA sequencing.

Sequence alignments

The alignments of genomic sequences and calculations of protein identities and similarities were done using Vector NTI, AlignX 2003 provided by InforMax Inc. (MD, USA). Alignments of protein sequences were done using ClustalW (Thompson et al., 1994).

Production of Fab1 antibody

Polyclonal antibodies against the N-terminal and the C-terminal parts of the Fab1 protein were generated using the sequence of an EST cDNA clone GH01668. A fragment encoding amino acids 1-400 (N-terminal part of protein) and amino acids 1424-1809 (C-terminal protein) were amplified by PCR. In the N-terminal protein a stop codon behind serine 400, an EcoRI site at the 5' end and a SalI site at the 3' end were introduced using sense and antisense primers; 5'CCGGAATTCATGACTAGCAACAACCAAAAC 3' and 3'GATATTCAAGAGGTTTTCGACTCAGCTGCAGC5' respectively. In the C-terminal protein an EcoRI site in front of glutamine 1424, at the 5' end and a stop codon in front of a SalI site at the 3' end were introduced using sense and antisense primers; 5'CCGGAATTCGAAGACAGTCCAAGCCTTTG 3' and 3'CCCTCCCGGAAAGGTTTCAGATTACTCAGCTGCAGC5' respectively. The obtained PCR product was run on an agarose gel, excised and extracted by QIAquick® Gel Extraction kit. The purified PCR product was then restricted with EcoRI/ SalI and cloned into EcoRI/ SalI digested vector pMAL-C2 to make a maltose binding protein (MBP)-fusion. Vector pMAL-C2 was transformed into DH10α cells

and plated out on ampicillin plates (100 µg/ ml). The plasmids were purified using procedures described above. The vector was further transformed into BL21 bacteria, incubated for 4 hours. IPTG (to final conc. 0,3 mM) was added to express the MBP-fusion protein in front of Ptac promoter and Fab1 N-term MBP-fusion protein was extracted (procedure described below). Proteins were dialysed ON in a PBS buffer and sent for immunisation in rabbits (EUROGENTEC S.A. Herstal, Belgium).

MBP fusion protein production and purification

A 5 ml pre-culture of LB containing ampicillin (100µg/ml) and transformed BL21 bacteria was set up ON and used to start a 500ml LB/ ampicillin culture. Bacteria were grown to an OD (600 nm) of 0.5, at after which point IPTG was added (final concentration 0.3 mM) to induce protein expression and the culture incubated to an OD of 1.5. Bacteria were centrifuged for 10 min at 5000 rpm (2,600 x g) (Eppendorf centrifuge 5417c, as described before). The supernatant was removed and the sample was frozen at -20°C. The bacterial pellet was dissolved in 20 ml sonication buffer (NaCl 200 mM, Tris 20 mM, DTT 1 mM protease inhibitor (EDTA (ethylene-diamine-tetra-acteic acid)-free protease inhibitor was used to dissolve the bacteria expressing the N-term protein containing the FYVE domain) and sonicated on ice 5 times (30 seconds, amplitude 50-60, pause 30 seconds in between). 1 ml Triton X-100 was added, the sample was left at shaking 4°C for 30 min and centrifuged at 12000 rpm (16,000 x g) for 15 min at 4°C (16,000 x g, Eppendorf centrifuge 5417c, as used before). Amylose resin in a 20 ml Bio-Rad Polyprep® Chromatography column (CA, USA) was pre-washed with 100 ml sonication buffer. The protein-containing supernatant with protein was removed and diluted in 100 ml sonication buffer. The supernatant was added to the column, but only the MBP-Fab1 fusion protein was bound to amylase beads. The MBP-fusion protein was eluted by addition of 20 ml sonication buffer with 10 mM maltose. Protein concentration was calculated by measuring OD (280 nm). OD was measured on a Hewlett Packard 845X UV-Visible System provided with UV-visible ChemStation software.

Antibody purification

MBP fusion proteins of C- and N-terminal parts of Fab1 were obtained as previously described. 1 mg of each protein was applied to a pre-washed Affi-gel® (Active Ester Agarose) 10 or 15 (depending on pI (isoelectrical point)) for binding and centrifuged at 10000 rpm (11,000 x g, Eppendorf centrifuge 5417c, as used before) for 3 min. The protein was incubated for 3.5 hours at 4 °C on a rotator. Glycine, pH 8, was added to a final concentration of 0.1 M (1 µM $ZnCl_2$ was added to the FYVE-domain containing N-terminal protein). The gel was incubated at RT for 1 hour, centrifuged at 10000 rpm (11,000 x g, Eppendorf centrifuge 5417c, as used before) for 3 min and washed 3 times with hepes buffer (20 mM hepes pH 7.2, 140 mM NaCl, 1 µM $MgCl_2$, 1 µM $ZnCl_2$, 1 mM DTT). The gel was washed 3 times with PBS, centrifugated at 10000 rpm (11,000 x g) for 1 min and incubated with 2 ml rabbit serum, third bleed, for 2 hours at RT. The gel was transferred to a 10 ml Bio-Rad Polyprep® chromatography column and washed with 20 column volumes of PBS. The purified antibody was eluted with 1 ml 0.1 M glycine pH 2.8 into tubes containing 25 µl 3 M tris pH 8.8. Glycerol was added 1:1 and the purified antibody stored at –20 °C.

Western blotting

Larvae were homogenised and mixed with concentrated samplebuffer. In the cases where single larvae were used, each individual larvae was homogenised and put directly into 20 µl SDS-PAGE 4X sample buffer (300 mM tris pH 6.8, 30 % glycerol, 10 % SDS, 0.6 % bromphenol blue, 600 mM DTT), 20 µl distilled water and 1.6 µl protease inhibitor. In other cases 20 larvae were homogenised together and centrifuged 1 min to remove cuticles (20,800 x g, Eppendorf centrifuge 5417c). The protein concentration was measured using a Bio-Rad protein assay, dye reagent concentrate and Biotrack II plate reader, (Amersham Biosciences, Oslo, Norway) following instructions provided by the supplier and approximately 30 µg protein was denatured with 4X sample buffer. Prior to loading on to a 7.5 % polyacrylamide gel, samples were heated to 100 °C for 10 min. For polyacrylamide gel electrophoresis,

standard procedures were used as described in (Sambrook, 2001) and protein standard Precision Plus Protein™ Standards Dual color was used to indicate protein sizes. Resolved proteins were transferred to Immobilon-P PVDF transfer membrane (Millipore Corporation, Bedford, MA) using a Mini Trans-Blot® Electrophoretic Transfer Cell (Bio-Rad, CA, USA) following the instructions provided by the supplier. After the protein transfer, the membranes were dried and blocked by incubation for 1 hour at RT, stained with Ponceau S Red solution (0.2 % Ponceau, 3 % trichloroacetic acid) and destained with distilled water. The membranes were re-soaked for 0.5 hour in PBS and 0.1 % tween (3 times change of solution) and incubated with primary antibody #267 diluted in PBS, 0.1 % tween and 2 % dry milk ON at 4 °C. The membranes were washed 3 times for 10 min with PBS and 0.1 % tween and incubated with secondary antibody (horse radish peroxidase (HRP) conjugated IgG HRP-goat anti-rabbit) diluted in PBS, 0.1 % tween and 2 % dry milk ON at 4 °C. The membranes were washed as before and developed in Super signal® West ECL (enhanced chemiluminiscence system) reagents following the instructions provided by the supplier.

Oregon green dextran (OGD) chase experiment in Garland cells

Wandering third instar wild type and *fab1* mutant larvae were collected and dissected to expose the Garland cells to the medium. The tissue was left in OGD diluted in Schneider medium (1:1) for 5 min, before incubation in lysotracker (1:1000) for 1 min, the Garland cells were mounted on a glass slide in 80% glycerol in PBS and covered with a coverslip. The tissue containing the cells for the chase experiment were washed and incubated in Schneider medium for 40 min before being exposed to lysotracker (1:1000 in PBS for 1 min) and mounted. All experiments were performed at 25 °C.

Antibody staining of imaginal discs

10-20 wandering third instar larvae were collected in a dissecting dish with PBS (140 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.1 mM NaH_2PO_4). The larvae were cut in

half with a pair of forceps and the anterior parts containing the imaginal discs were inverted. The anterior body parts of the larva were transferred to a 24 well tissue culture plate (Labware, New Jersey) containing fixative (PBS, 0.1% Triton X-100 and 3% formaldehyde (FA)) and incubated on ice for 20 min. The tissue was washed 5-6 times (30 min) with PBX (PBS and 0.1% Triton X-100) and blocked for 1 hour in PBX+ 0.1% bovine serum albumine (BSA). The tissue was incubated with primary antibody #267 diluted in PBX, 0.1% BSA and 5% goat normal serum (GNS) ON and washed as before. The tissue was incubated ON with Cy2 conjugated secondary antibody diluted as described above. The tissue was washed as before and covered in 80% glycerol in PBS and 0.4% n-propylgallate, before ON storage at 4°C. Discs were dissected out and mounted onto a glass slide, covered with a coverslip, and sealed with nail varnish.

Embryo collection and staining of PNS

The embryos were collected after two hours egg laying on apple juice plates (22.5 g agar, 750 ml dH_2O, 25 g sucrose, 250 ml apple juice, 1.5 g nipagin dissolved in 3 ml ethanol) with dried yeast and aged to stage 16 (15 hours). The embryos were dechorionated with 6.5% sodium hypochlorite by gently rocking the plate for 1 min, then transferred to a filter container and rinsed in distilled water. The embryos were transferred with a spatula to an Eppendorf tube containing freshly made fix solution (heptane 500 µl, PBS 450 µl, 50 µl 16% FA), vortexed to mix and incubated for 20 min on an orbital shaker. The lower, aqueous phase was removed; excess 100% methanol was added and mixed well by inversion. The upper heptane phase was removed, more methanol added and mixed by inversion. The dechorionated embryos collected at the bottom of the tube and were washed once more in methanol. The methanol was removed, excess PBX was added and the embryos were rinsed twice in PBX. The embryos were blocked in PBT (PT, 0.5% BSA) for 30 min at an orbital shaker. The embryos were incubated with primary antibody 22C10 and rabbit polyclonal anti β-Galactosidase, diluted in PBT and 5% GNS ON on a rocking table at 4°C. The embryos were washed 3 times in PBT for 5 min, twice for 20 min and

incubated with Cy3 conjugated IgG secondary antibody diluted in PBT for 2 hours at room temperature, covered from light on a rocking table. The embryos were washed 3 times for 5 min and twice for 20 min in PBT and mounted in 80% glycerol. The embryos were visualised by immunofluorescence microscopy.

Confocal immunofluorescence microscopy

All fluorescence microscopy was carried out on a Zeiss LSM 510 confocal microscope from Zeiss (Jena, Germany). Images were processed with Adobe photoshop from Adobe (San Jose, CA) and Zeiss LSM Image Browser (Version 3) from Zeiss (Jena, Germany).

Appendix 1

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mofab1          -------------------MATDDKSSPTLDSANDLPRSPASPSHLTHFKPLTPDQDEPP 41
drfab1          ------------------------MTSNNQNNSSSHQHLHSPSKLTEFARNFEDKPESL 35
cefab1          ------------------------------------------------------------
yfab1           MSSEEPHASISFPDGSHVRSSSTGTSSVNTIDATLSRPNYIKKPSLHIMSTSTTSTTTDL 60

mofab1          FKSAYSSFVNLFRFNKERGEGGQGEQQSPS----------------SSWASPQIPSRTQS 85
drfab1          FGRVVNKIQNVYNQSYNTVNDISSGSSSSSSTQPVQVVGKSQFFSDSQTSTAEIADVETS 95
cefab1          ------------------------------------------------------------
yfab1           VTNPILSNISVPKISPPTSSSIATATSTSHVTGTASHSNIKANANTSTSVNKKNLPPTTS 120

mofab1          VRSPVPYKKQLNEELHRRSSVLENTLPHPQESTDSRRKAEPACGGHDPR----------- 134
drfab1          SQSSVRPQPPTTLSIRTNSETRGTSTSSNTAAEDSETSDRVETLPLPTS----------- 144
cefab1          ------------------------------------------------------------
yfab1           GRIPSSTIKRYPSRYKPSHSLQLPIKNDSNFKRSSIYASKSTVTAIPIRNNRPISMQNSY 180

mofab1          ------------------TAVQLRSLSTVLKRLKEIMEGKS-------QDSDLKQYWMPD 169
drfab1          ------------------EANQGRTVSNVLKHISNIVATKNNNDLRNYKDTELQRFWMPD 186
cefab1          ------------------------------------------------------MPD 3
yfab1           ARTPDSDHDDVGDEVSSIKSASSSLTASLSKSFLFAFYNNRKKDKTSNNGVLSKEYWMKD 240
                                                                       * *

mofab1          SQCKECYDCSEKFTTFRRRHHCRLCGQIFCSRCCNQEIPGKFMGYTGDLRACTYCRKIAL 229
drfab1          SKAKECYDCSQKFSTFRRKHHCRLCGQIFCSKCCNQVVPGMIIRCDGDLKVCNYCSKIVL 246
cefab1          STGRECYQCEERFTTFRRRHHCRLCGQIFCAKCCSSHIDGAALGYMGELRLCDYCARKVQ 63
yfab1           ESSKECFSCGKTFNTFRRKHHCRICGQIFCS-SCTLLIDGDRFGCHAKMRVCYNCYEHAD 299
                .  :**:.* : *.****:****:******: .*.  : *  :   ..:: *  * . .

mofab1          SYAHSTDSNSIGEDLNALSDSTCSVSILDPSEPRTPVGSRKASRNIFLE----------- 278
drfab1          TFLKSS-SSEMGQDMQELQQHLSNKLEVQ------------------------------ 274
cefab1          RLAEEGKQTPTTSTTRSQTPVNSRKISFDRN---------------------------- 94
yfab1           TYEDSSDEENDSTMQLNEPRSRSRSRSSNTNPYSHSHSHLHLISQDNHNGTDLHDPVAAT 359
                   ..  .              .     :

mofab1          ------------------------------------------------------------
drfab1          ------------------------------------------------------------
cefab1          ------------------------------------------------------------
yfab1           DNPQQQNEVYLLNDDDVQSIMTSGEDSKLFISTPPPPPKMAIPATKQGGSLEISFDSEND 419

mofab1          -------DDLAWQSLIHPDSSNSALSTRLVSVQEDAGKSPARNRSASITNLSLDRSGSPM 331
drfab1          ---------------------------------DSGSSLAKHP-------QMQRAPLPR 293
cefab1          --------------------------------TAHKNSDTVRTVSNGAIWSLCPPESSM 121
yfab1           RALHYQDDNPGRHHHLDSVPTRYTIRDMDNISHYDTNSNSTLRPHYNTNNSTITINNLNN 479
                                             .. : .         :

mofab1          VPSYET----------------------------SVSPQANRNYIRTETTEDERKILLD 362
drfab1          KTSVGY----------------------------QEERFSSHPTYTTLSIDDRKNILQQ 324
cefab1          PPEITS----------------------------PPQLGSRRNSLAQSSNGPGVPTILS 152
yfab1           TTSNNSNYNNTNSNSNINNPAHSLRRSIFHYVSSNSVNKDSNNSSATPASSAQSSSILDP 539
                 ..                                .  : .      :       :

mofab1          SAQLKDLWKKICHHTSGMEFQDHRYWLRTHPNCIVGKELVNWLIRNGHIATRAQAIAIGQ 422
drfab1          SNSLITLHEEMQRDLP--------------AQNCGQRLIEFLNSNNKSANEVQAVAILN 369
cefab1          VADLCASNSAMLTNSHS-------------------------HPMITEEEESGPDWFR 185
yfab1           ANRIIGNYAHRNYKFKFNYNSKGPSQQNDTANGNNDNNNNNNNNNNNNNNNSASGIADNN 599
                   :         .                                      ..    .

mofab1          AMVDGRWLDCVSHHDQ-------LFRDEYALYRPLQSTEFSETPSPDSDSVNSVEGHSEP 475
drfab1          AMLAAGFLEPIVPDPE-------QMDFDSSLHYKFSKSSSSDTSRTMSPQFEANP-HAEP 421
cefab1          TMHPG--MDGVINNET-------SDSADVFAYANLAGAITNEFTEMMDARAAEPTSATTD 236
yfab1           NIPSNDNGTTFTLDKKKRNPLTKSKSTSAYLEYPLNEEDSSEDEGSMSIYSVLNDDHKTD 659
                 :        .  .             .      :     .:     .

mofab1          SWFKDIKFDDSDTEQIAEEGD---------------------------------------- 496
drfab1          QPPKSMDQSAEEKEKELEN----------------------------------------- 440
cefab1          RKITFPSLSLDESVMNAAK------------------------------------------ 255
yfab1           NPIRSMRNSTKSFQRAQASLQRMRFRRKSKSKHFPNNSKSSIYRDLNFLTNSTPNLLSVV 719
                       . ..      .
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mofab1          --DNLAKYLVSDTGGQQLSISDAFIKESLFNRRVEEKSKELPFTPLGWHHNNLELLREEN 554
drfab1          ----------------------------------------------------ELENDR 446
cefab1          ------------------------------------------------------------
yfab1           SDDNLYDDSSPLQDKASSSAASRLTDRKFSNSSGSNNNSNSNSNINTDPWKRIASISGFK 779


mofab1          EEKQAMERLLSANHNHMMALLQQLLQNESLS-SSWRDIIVSLVCQVVQTVRPDVKHQDDD 613
drfab1          CYTTATSKLLASYCEHEEQLLAQMLRAHNLD-QEWDKVLQMLCSTAANHFKPEHCSN-DL 504
cefab1          --EEQRDNLEELFRRNTERILDEVMKREYIREDKWRDLILKSVYEVVENVTVNVPSG-DT 312
yfab1           LKKEKKRELNEVSLLHMHALLKQLLNDQEISNLQEWITLLDGALRKVLRTILNARDLNTL 839
                       .*      :   :* :::. . :   .    :       .     :


mofab1          MDIRQFVHIKKIPGGK-KFDSVVVNGFVCTKNIAHKKMN----SCIKNPKILLLKCSIEY 668
drfab1          MDIRNYVNFKKVPGGR-RKDSKIVHGVAFSKNVAHKDMA----THVPFPRILLLQCPIVY 559
cefab1          MNIADYVHVKKVHKKEGKVDSEIIWGVACSRSLVYKSLSEEDESSHTTESIMIVSGSIEY 372
yfab1           DFRQTYVKIKRISGGS-PQNSEYIDGVVFSKALPSKTMP----RHLKNPRILLIMFPLEY 894
                     :*:.*::       :*  : *.. :: :  * :            *:::  .: *


mofab1          LYREETKFTCIDPIVLQEREFLKNYVQRIVDVRPTLVLVEKTVSRIAQDMLLEHGITLVI 728
drfab1          ER-IEGKFVTIETVLLQEKEYLRNVCARIMSFKPNVVLVHKNVAGIAQDLLRSYEVTLVL 618
cefab1          ER-VSNKLSSIEPIIVQEEKFLEKQIDRIATKRASLILVEGGVSHIAAQLLHKRGIKVAV 431
yfab1           QK-NNNHFLSIESVFRQEREYLDKLVSRLKSLHPDIIYVGANVSGYALELLNDSGIVVQF 953
                    . ::  *:.:. **.::* :   *:   :. :: *   *:  * ::* .  : : .


mofab1          NVKSQVLERISRMTQGDLVVSMDQLLTKPHLGTCHKFYMQIFQLPNEQTKTLMFFEGCPQ 788
drfab1          DVKLSVMERLSRTLQCDIVSSIESNITMPKLGYCNDFYIRNYNG-----KTLMFFEKLTN 673
cefab1          NVKMSILQRISRATGADIVSNSDSQLVEQNLGCCPEFQQRNMQQEDGRIKTLMIFADCQK 491
yfab1           NMKPQVIERIAKLTEADIAISVDKLATNIKMGECETFEVKSYIYGN-ISKTYTFLRGCNP 1012
                ::* .:::*:::    *:. . :.  .  ::* *  *  :          **  ::


mofab1          HLGCTIKLRGGSDYELARVKEILIFMICVAYHSQLEISFLMDEFAMPPTLMQSPSFHLLT 848
drfab1          PRGYTCLLRGGSNAELTRVKRVASALLFARYNWRLEMSFLLNEFAQP------------- 720
cefab1          ETGCTVLLHGDDLKELVAVKRVVQFLVTIVYSNYLEQSYLN-------------------- 532
yfab1           ELGGTILLRGDSLENLRKIKQVSEFMVYAIFSLKLESSFFNDNFIQLSTDVYLKRAESKK 1072
                  * *  *:*..  :*  :*.:   ::   :   ** *::


mofab1          EGRGEEGASQEQVSGSSLPQDPECPREALSSEDSTLLESRTVLEKGELDNKSIPQAVASL 908
drfab1          ------------------------------------LSPKPSIFDSKETSPKTETEAELRS 745
cefab1          ----------------------------------------AFNTTIARRQSDCVVCE 549
yfab1           LQVFEG-YFADFLIKFNNRILTVSPTVDFPIPFLLEKARGLEKKLIERINQYESESDLDR 1131
                                                                :  .:.


mofab1          KHQDYTTPTCPAGIPCALFALVPESLLPLHMDQQDAVGNEHRETSQQTDEQQDPKSQMKA 968
drfab1          KRPIILERKSEDKITTIVSENVSDFTDPLRASQAEALSTSPCAPPVVEALAVEPR----- 800
cefab1          KRRAIVYSQGEK-------------------------------------------------- 561
yfab1           QTQLNMLQGLESTITKKHLGNLIKFLHEMEIENLELEFQKRSRQWEVSYSSSQNLLGTGS 1191
                :


mofab1          FRDPLQDDTGMYVTEEVTSSEDQRKTYALTFKQELKDVILCISPVITFREPFLLTEKGMR 1028
drfab1          --------------------------YDNRFRTALSSTLLSVSPFLTFPLPYLETEQGRK 834
cefab1          ---------------------------TEFEKNLYATMLSSSPVIEFEPPLLETATGRE 593
yfab1           HQSITVLYSMVSTKTATPCVGPQIVTIDYFWDSDISIGQFIENVVGTARYPCQQGCNG-- 1249
                                          :   :    :  . .     *      *


mofab1          CSTRDYFPEQIYWSPLLNKEVKEMESRRKKQLLRDLSGLQGMNGSVQAKSIQVLPSHE-- 1086
drfab1          CKLRKLFPAELYFSKQWSRTGLERPDSMGD------GEAGKSEPGNKENQMQLLPAHD-- 886
cefab1          CPLIAYFKQPLYKLLKP------------------GDVELIKQGYEEDIVPIPKKEP-- 632
yfab1           -LYLDHYRSYVHGSGKVDVLIEKFQTRLPKLKDIILTWSYCKKCGTSTPILQISEKTWNH 1308
                      :   ::                              : . .   : :


mofab1          ----LVSTRIAEHVGDSQTLGRMLADYRARGGEFSQNIWNPFVHSKDDIMYFRWQIRETK 1142
drfab1          ----FVLMKITAPAS-SRDIQSKLAEFRSFGGRLPKGKAPMLRPKKKNAEVIQRPQKVSE 941
cefab1          ----LLVDRRHAFAQCNRGIN--------------------FRRRTAQIVKHRKIVETE 667
yfab1           SFGKYLEVMFWSYKDSVTGIGKCPHDFTKDHVKYFGYNDLVVRLEYSDLEVHELITPPRK 1368
                     :            :                                .     :


mofab1          LRVMKERGLIPSDVIWPTKVDCLNPANHQRLCVLFSSSSAQSSNAPSACVSPWIVTMEFY 1202
drfab1          EQLYK--------------DALDPQNHQRLPVLFCSFHYNPKGVSSFCKLPMLLDMKFY 986
cefab1          KQPFR------------AKDVLDPRVHQTLAVLFGSFSRKSPNAPYFCVRPWVVSMQYY 714
yfab1           IKWKPHIDIKLKVELYYKILEKINNFYGSVLSRLERIKLDSMTKDKVLSGQAKIIELKSN 1428
                 :                 : ::    . *  *      .       .  . :: ::


mofab1          GKNDLTLGIFLERYCFRYSYQCPSMFCDTPMVHHIRRFVHGQGCVQIILKELDSPVPGY- 1261
drfab1          GQYDIMLEQFLQRYCCLFNSMCP--SCNLPMLGHVRRYVHSLGCVHVYLTEDLTRSD--- 1041
cefab1          KDHDMTIGEFLVKFCFNRSYECPSSNCEVPMLDHSRKLVYGKVCVEISTQTVNEAENAIE 774
yfab1           ATEEQKLMLQDLDTFYADSPCDQHLPLNLVIKSLYDKAVNWNSTFAIFAKSYLPSETDIS 1488
                   :  :           .        :  :     : *     . :


mofab1          ---QHTILTYSWCRICKQVTPVVALSNESWSMSFAKYLELRFYGHQYTRRAN-------A 1311
drfab1          ---PTRIYFTSWCSICNATTPTIPLSDAAKCLSLAKYLEMRFHGHAYKRRPPSTDAEQGG 1098
cefab1          SEQQKSIMTWRNCGKCNCSSQMVKFDKAIWHLSFAKFLEYIGNSCFTTDTIYPITN---Q 831
yfab1           RITAKQLKKLFYDSSRKDSEDKKSLHDEKAKTRKPEKNELPLEGLKDVEKPKIDSKNTTE 1548
                      :         :       : .       .:  *    .
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```
mofab1          EPCGHSIHHDYHQYFSYNQMVASFSYSPIRLLEVCVPLPKIFIKRQAPLK---------- 1361
drfab1          TVCEHSLHRDYVHHFSFRGVGAKFQYTPVEVWETDLPSLTVQLDLPQPFQS--------- 1149
cefab1          NQCSHCFFHEKLYFFAMDNLVTTFKVIAIRPYSVVFSPIICSVKVLKVSR---------- 881
yfab1           NRDRTNEPQNAVTITTFKDDTPIIPTSGTSHLTVTPSASSVSSSLTPQTEERPPISRSGT 1608
                        ::     :     . :         .  .      .     .

mofab1          -VSLLQDLKDFFQKVSQVYLAVDERLASLKTDTFSKTREEKMEDIFAQKEMEEGEFKNWT 1420
drfab1          -AQVQEEIKNFSIKGHEVYNRIHERIADLATEEENSPLVQHLKTMLTHDQFIFKQKIEIV 1208
cefab1          -KELSDDVSRIATLALTACEDTNKQLAELDEEVQITPIVVKLAGAIRNTMALASESRMFA 940
yfab1           GISMTHDKSTRPNIRKMSSDSSLCGLASLANEYSKNNKVSKLATFFDQMHFDALSKEFEL 1668
                  .: .: .               :*.*  :   .    ::   : :      .

mofab1          EKMQARLMSSSVDTPQQLQSIFESLIAKKQSLCEVLQAWNSRLQDLFQQEKGRKRPSVPP 1480
drfab1          HTLLTDNRATAYDT-------SDALAMARRALAESIELWGPRLQEIEK------------ 1249
cefab1          KNILS---------------------------------------------------- 945
yfab1           ERERERLQLNKDKYQAIRLQTSTPIVEIYKNVKDAVDEPLHSRSSGNNLSSANVKTLEAP 1728
                .

mofab1          SPGRLRQGEESKINAMDTSPRNISPGLSQWRKRRSLLDNPVQPATSSTHLQLPTPPEALA 1540
drfab1          ----LTAKQAHHIDSGTICTEELRP--------EQVQTADSSKVTTSSLPKENDPLECPS 1297
cefab1          -------GDEDLIRSNDRLYREVTG---------TFMKVREVTYNLIALWNENCAAIKYP 989
yfab1           VGEHSRANNCNPPNLDQNLETELENSISQWG---ENILNPSGKTTASTHLNSKPVVKETS 1785
                        :            ::                    .  :  :        .

mofab1          EQVVGGPTDLDSASG--SEDVFDGHLLGSTDSQVKEKSTMKAIFANLLPGNSYNPIPFPF 1598
drfab1          EDTETGASNSQTVLD--KNFSIDQMLASTVNVYSDKKSIRKILTQLLPSGNQVNPLQSPF 1355
cefab1          KRTPEDIQEIATLQK--LENPFPSHLHLAIKLQPRLGVVVRDIQDTRGN----------- 1036
yfab1           ENPKSIVRESDNSKSEPLPPVITTTTVNKVESTPQPEKSLLMKTLSNFWADRSAYLWKPL 1845
                :       :  .         :        .

mofab1          DPDKHYLMYEHERVPIAVCEKEPSSIIAFALSCKEYRNALEELSKATLRNSAEEGLPANS 1658
drfab1          -PAQDHLTLPLGSIPIHVRETDLSSVIAYSLTSMDYQKAIDEAEANSNAAHSSPQLKRKI 1414
cefab1          ------------------FKPDIGSIIAYALSAVDYNKIPEAADTVSMDSASSSLKFSQM 1078
yfab1           VYPTCPSEHIFTDSDVIIREDEPSSLIAFCLSTSDYRNKMMNLNVQQQQQQQTAEAAPAK 1905
                                  : : .*:**:.*:  :*.:     .


mofab1          ALDNRPKSS-SPIRLPEISGGQTNRTVEAEPQPTKKASGMLSFFRGTAGKSPDLSSQKRE 1717
drfab1          PLAESVSDAEDSPSLSRTSSNTSAAPNASVPSPATAASESEEKSKERIKQPPSPHITLAF 1474
cefab1          DDGENLASS---------------------------------------------------- 1087
yfab1           TGGNSGGTT-----------------QTGDPSVNISPSVSTTSHNKGRDSEISSLVTTK 1947
                   :    :

mofab1          TLRGADSAYYQVGQAGKEGLESQGLEPQDEVDGGDTQKKQLTNPHVELQFSDANAKFYCR 1777
drfab1          QDHSCQFQCKIYFAREFDAMRSKSLKPP-KLDKSLYRRLEKSKMREELRISQSRTGSEME 1533
cefab1          ----------------------------------------QHLEVEFEDESASYYVK 1104
yfab1           EGLLNTPPIEGARDRTPQESQTHSQANLDTLQELEKIMTKKTATHLRYQFEEGLTVMSCK 2007
                                                       : . .:.:  :    .

mofab1          LYYAGEFHKMREVILGSSEEE------FIRSLSHSSPWQARGGKSGAAFYATEDDRFILK 1831
drfab1          LVRKPSDVGAPRTTEDDSNQEEDARIALARSLCKSVQWEARGGKSGSRFCKTLDDRFVLK 1593
cefab1          MLYAEKFRKLRELLIAEGEET------FIRSLSNSTFWTPQGGKSGSFFYRTQDDRFVVK 1158
yfab1           IFFTEHFDVFRKIC--DCQEN------FIQSLSRCVKWDSNGGKSGSGFLKTLDDRFIIK 2059
                :          .    . ::       : :**...  * ..*****: *  * ****:.*

mofab1          QMPRLEVQSFLDFAPHYFNYITNAVQQKRPTALAKILGVYRIGYK-NSQNNTEKKLDLLV 1890
drfab1          EMNSRDMTIFEPFAPKYFEYIDRCQQQQQPTLLAKIFGVFRVSVK-KKDSFVER--SVMV 1650
cefab1          QMSRFEIQSFVKFAPNYFDYLTTSATESKLTTLCKVYGVFRIGYK-SKT--TTLKVDILV 1215
yfab1           ELSHAELEAFIKFAPSYFEYMAQAMFHDLPTTLAKVFGFYQIQVKSSISSSKSYKMDVII 2119
                ::   ::  *  *** **:*:  .  ..  * *.*: *.:::  * .         .:::

mofab1          MENLFYGRKMAQVFDLKGSLRNRNVKTDTGKESCDVVLLDENLLKMVRDNPLYIRSHSKS 1950
drfab1          MENLFYGCNIENKFDLKGSERNR--LVDPSNQQGEIVLLDENLVQMSWSKPLYVLSHSKT 1708
cefab1          MEYLFYNHNVSQVWDLKGSLRNR--LASTGKSANEMVLLDENFVKDLWNQQLYVLPHSKA 1273
yfab1           MENLFYEKKTTRIFDLKGSMRNR---HVEQTGKANEVLLDENMVEYIYESPIHVREYDKK 2176
                ** ***  :  . :***** ***       .   : ******:::   .. :::  :.*

mofab1          ELRTSIHSDAHFLSSHLIIDYSLLVGRDDTSNELVVGIIDYIRTFTWDKKLEMVVKSTGI 2010
drfab1          VLRDAIQRDSSFLEKNLVMDYSLLVGLDKKNGVLVLGIIDYIRTFTLDKRVESIIKGSGI 1768
cefab1          AMNQAISNDSHFLSSQYIMDYSLLVGVDDDNGELILGIVDYMRTYTLDKKLESWVKIVAI 1333
yfab1           LLRASVWNDTLFLAKMNVMDYSLVIGIDNEGYTLTVGIIDFIRTFTWDKKLESWVKEKGL 2236
                 :. ::  *: ** .  ::****::* *. .  * :**:*::**:* **::*  :*  .:

mofab1          LGGQ--GKMPTVVSPELYRTRFCEAMDKYFLMVPDHWTGLDLNC-- 2052
drfab1          LGGK--GKDPTVVNPERYKQRFIDAMDRYFLTVPDRWEGLSKV--- 1809
cefab1          PG----AHLPTILSPEMYCARFSEAIDSYFPVVPDQWTGLGSIRSY 1375
yfab1           VGGASVIKQPTVVTPRQYKKRFREAMERYILMVPDPWYWEGN---- 2278
                 *     : **::.*. *  ** :*:: *:  *** *   .
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Appendix 1. Alignment (ClustalW (Thompson et al., 1994)**) of *Mus musculus* (AF102777), *Drosophila melanogaster* (CG6355), *Caenorhabditis elegans* (T18961) and *Saccharomyces cerevisiae* (YFR019W) Fab1 orthologs (accession numbers in parentheses).** Database analysis with Pfam (Bateman et al., 2004) of the *D. melanogaster* sequence showed domains similar to a PI(3)P binding FYVE domain (amino acids 181-247) located at the N-terminus of the protein (Ponting and Bork, 1996), a chaperonin-like region, Cpn60_TCP1 (amino acids 424-824), found in proteins implicated in actin and tubulin folding, and a putative catalytic kinase domain (amino acids 1563-1796) all underlined and shaded in grey (Shisheva, 2001).

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