Drosophila 14-3-3ε has a crucial role in anti-microbial peptide secretion and innate immunity

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Summary

The secretion of anti-microbial peptides is recognised as an essential step in innate immunity, but there is limited knowledge of the molecular mechanism controlling the release of these effectors from immune response cells. Here, we report that *Drosophila 14-3-3ε* mutants exhibit reduced survival when infected with either Gram-positive or Gram-negative bacteria, indicating a functional role for 14-3-3ε in innate immunity. In *14-3-3ε* mutants, there was a reduced release of the anti-microbial peptide Drosomycin into the haemolymph, which correlated with an accumulation of Drosomycin-containing vesicles near the plasma membrane of cells isolated from immune response tissues. Drosomycin appeared to be delivered towards the plasma membrane in Rab4- and Rab11-positive vesicles and smaller Rab11-positive vesicles. RNAi silencing of Rab11 and Rab4 significantly blocked the anterograde delivery of Drosomycin from the perinuclear region to the plasma membrane. However, in *14-3-3ε* mutants there was an accumulation of small Rab11-positive vesicles near the plasma membrane. This vesicular phenotype was similar to that observed in response to the depletion of the vesicular Syntaxin protein Syx1a. In wild-type *Drosophila* immune tissue, 14-3-3ε was detected adjacent to Rab11, and partially overlapping with Syx1a, on vesicles near the plasma membrane. We conclude that 14-3-3ε is required for Rab11-positive vesicle function, which in turn enables antimicrobial peptide secretion during an innate immune response.

Key words: Innate immunity, Vesicular traffic, 14-3-3, Antimicrobial peptide, Drosomycin, Secretion, *Drosophila*

Introduction

Innate immunity is the crucial first line of defence against microbial challenge. The genes involved in innate immunity encode microbe-fighting peptides and cytokines, which can kill pathogens and induce inflammation. When unchecked these inflammatory mediators can cause septic shock and promote autoimmune diseases, such as multiple sclerosis, type 1 diabetes mellitus and rheumatoid arthritis (Grateau, 2006). Anti-microbial peptides (AMPs) are activated by the two major innate immunity pathways, the Toll–interleukin-1 (IL-1) pathway and the immune-deficiency (IMD)–tumour necrosis factor α (TNFα/Eiger) pathway, which are highly conserved in evolution, from insects to humans (Lemaitre and Hoffmann, 2007; Leulier and Lemaitre, 2008). This species conservation makes *Drosophila* a valuable model to study the molecular events that regulate the innate immune response.

The generation of an innate immune response in *Drosophila* is mediated by the fat body (the humoural response tissue, analogous to the vertebrate liver), blood cells called haemocytes (the cellular response) and other tissues, such as the gut and trachea, which form a barrier between the environment and the living organism (Ferrandon et al., 2007; Hultmark and Borge-Renberg, 2007; Lemaitre and Hoffmann, 2007). If this barrier is breached bacterial pathogens can enter the haemolymph, where they are recognised by specific secreted or membrane bound peptidoglycan-recognition protein receptors (PGRPs) and Gram-negative-binding proteins (GNBPs), which act to trigger either the Toll or immune deficiency (IMD) signalling cascades in immune response tissues. Microbial challenge activates the host fat body cells to secrete various anti-microbial peptides (AMPs), including Diptericin (Dpt) and Drosomycin (Drs) (Imler and Bulet, 2005). The secretion of these anti-microbial peptides is an essential step in the immune response.

Endocytosis and exocytosis are directly involved in the immune response in eukaryotes (Robatzek, 2007; Stow et al., 2006). For example, endosomal compartments contain internalised Toll and Toll-like receptors, which activate the downstream signalling cascades (Husebye et al., 2006). A final step in the immune response involves the exocytosis of immune response mediators, such as AMPs (Batoni et al., 2006; Maisetta et al., 2008; Urbe et al., 1993). Although some of the molecular events in anterograde protein trafficking from the Golgi to the plasma membrane have been described, the molecular machinery involved in regulating the final stages of vesicular exocytosis are yet to be fully elucidated. The small GTPases Rab4 and Rab11, which function in recycling endosomes, have been implicated in the anterograde traffic of soluble cargo. Rab11 is essential for vesicle targeting to the plasma membrane (Menager et al., 2007; Sonnichsen et al., 2000; Ullrich et al., 1996; Ward et al., 2005; Zerial and McBride, 2001). At the plasma membrane, these Rab11-positive vesicles are recognised by SNARE [soluble-*N*-ethylmaleimide-sensitive factor accessory-protein (SNAP) receptor] and Syntaxin proteins, which are involved in the final stages of exocytosis and secretion (Stow et al., 2006).

Some immune-deficiency disorders are known to be caused by the loss of function of proteins involved in the vesicular machinery. Mutations in endosomal proteins are associated with immune

diseases, such as Griscelli and Hermansky–Pudlak syndromes (Ménasché et al., 2000; Stow et al., 2006). In mammals, SNAREs are crucial for vesicle degranulation and the subsequent release of inflammatory mediators or cytokines, from the secretory granules in mast cells, eosinophils, neutrophils and platelets (Kay et al., 2006; Logan et al., 2006; Manderson et al., 2007; Stow et al., 2006). Patients with mutations in the SNARE protein Sec1/Vps33B exhibit bleeding problems associated with defects in megakaryocyte and platelet alpha-granule release (Lo et al., 2005). New elements of the vesicular secretion machinery are being realised on the basis of immune-deficiency disorders.

There have been several reports implicating 14-3-3 proteins in traffic to the plasma membrane (Shikano et al., 2005; Tzivion and Avruch, 2002; Tzivion et al., 2001). The 14-3-3 family of phosphorylated-serine-binding proteins is highly conserved from yeast to man, and in mammals comprises seven isoforms (β, γ, ε, η, σ, τ and ζ) (Tzivion and Avruch, 2002; Yang et al., 2006). 14-3-3 proteins are dimeric with homodimers and heterodimers formed between most isoforms (Messaritou et al., 2010). The 14-3-3 dimer structure comprises nine α-helixes arranged to form an internal pocket that provides a surface for interaction with a large number of phosphorylated-serine target proteins, some of which have been identified by proteomic analysis (Darling et al., 2005; Mackintosh, 2004; Pozuelo Rubio et al., 2004; Yang et al., 2006). Understanding the biological role of 14-3-3 proteins in vivo is extremely difficult given their functional redundancy in mammals. *Drosophila*, however, has only two 14-3-3 homologues, 14-3-3ε and 14-3-3ζ, which have 82% and 88% sequence identity to the respective mammalian orthologues and also exhibit functional conservation (Benton et al., 2002; Broadie et al., 1997; Li et al., 1997; Skoulakis and Davis, 1998; Su et al., 2001). Therefore, *Drosophila* represents a unique and tractable model in which to determine the in vivo function of 14-3-3 proteins. We have taken advantage of a *Drosophila* model, to show that 14-3-3ε plays an important role in innate immunity.

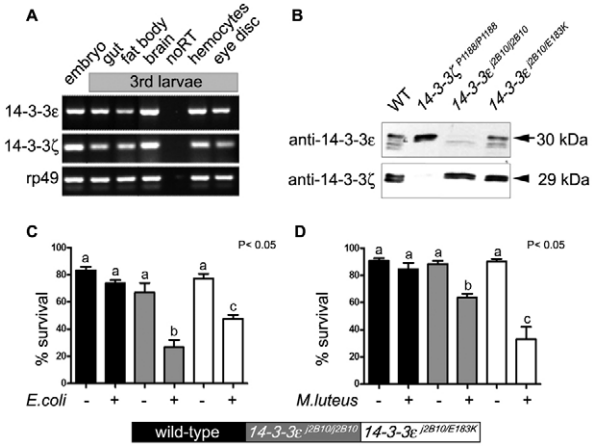

Fig. 1. *14-3-3*ε mutants showed decreased survival in response to Gram-negative and Gram-positive bacterial challenge. (**A**) RT-PCR analysis of *14-3-3*ε and *14-3-3*ζ mRNA expression in embryos and third-instar larval tissues (3rd larvae): gut, fat body, brain, haemocytes and eye imaginal disc. Negative control (lane 5; no RT), 1st strand reverse transcription reaction carried out without reverse transcriptase, confirming the specificity of primers for the respective cDNAs. Bottom panel: transcription of ribosomal gene *rp49* (also known as *RpL32*) used as an endogenous control. (**B**) Total protein extracts from wild-type third-instar larval fat bodies (WT, wild-type), late-stage 17 embryos mutant for *14-3-3*$\zeta^{P1188/P1188}$ and third-instar larval fat bodies from *14-3-3*$\varepsilon^{j2B10/j2B10}$ and *14-3-3*$\varepsilon^{j2B10/E183K}$ mutants were assessed for 14-3-3ε and 14-3-3ζ protein expression by western immunoblotting with either anti-14-3-3ε (upper panel) or anti-14-3-3ζ (lower panel) antibodies. (**C**,**D**) Histograms showing the percentage survival of wild-type (black bars), *14-3-3*$\varepsilon^{j2B10/j2B10}$ (protein null) homozygotes (grey bars) or *14-3-3*$\varepsilon^{j2B10/E183K}$ trans-heterozygotes (white bars) challenged with *E. coli* (+ in C) or *M. luteus* (+ in D). For each group, at least n=500 individual larvae were analysed and results are mean+s.e.m. One-way ANOVA analysis of variances showed significant differences between group means (P<0.05). Tukey's multiple comparison test showed significant differences between the means of genotypes depicted by the different letters on the bars (P<0.05).

Results

Loss of 14-3-3ε results in *Drosophila* susceptibility to acute bacterial infection

Both *Drosophila* 14-3-3ε and 14-3-3ζ are broadly expressed throughout development and their transcripts and proteins can be detected in most, if not all, embryonic and larval tissues (Fig. 1A). As previously described, the loss of 14-3-3ζ (Fig. 1B, lane 2) resulted in embryonic lethality even in the presence of functional 14-3-3ε (Acevedo et al., 2007). By contrast, neither *14-3-3*ε^{j2B10} homozygotes (protein null allele; Fig. 1B, lane 3) nor *14-3-3*$\varepsilon^{j2B10/E183K}$ trans-heterozygotes [protein null allele and antimorphic allele carrying the E183K point mutation (Benton et al., 2002); Fig. 1B, lane 4] showed impaired viability for the third-instar larvae when compared with wild-type larvae [Fig. 1C,D; see no bacterial treatment (–)]. Given that viability of *14-3-3*ε mutants was normal under steady-state conditions and that 14-3-3ε was detected in all immune response third-instar larval tissues (i.e. fat body, gut and haemocytes, Fig. 1A), we hypothesised that 14-3-3ε was essential for the immune response under conditions of challenge. To address this question we infected either wild-type or *14-3-3*ε mutant *Drosophila*, through the natural oral route. Early third-instar larvae were incubated at 29°C with a suspension of either Gram-negative *Escherichia coli* (*E. coli*) (for 11 hours, Fig. 1C) or Gram-positive *Micrococcus luteus* (*M. luteus*) bacteria (for 7 hours, Fig. 1D). The surviving larvae and pupae were scored at 6-hour intervals. Whereas the survival of wild-type *Drosophila* was not significantly affected by infection of either *E. coli* (survival of non-infected larvae was 83.3±2.6% compared with that of infected larvae at 73.7±2.6%) or *M. luteus* (non-infected survival of 90.8±1.8% compared with infected survival of 84.4±4.6%), the survival of *14-3-3*ε^{j2B10} homozygotes was significantly (P<0.05) reduced after infection with either *E. coli* (non-infected survival of 66.9±7.0% compared with infected survival of 26.7±5.3%) or *M. luteus* (non-infected survival of 88.4±2.3% compared with infected survival of 63.7±2.8%). Similarly, *14-3-3*$\varepsilon^{j2B10/E183K}$ trans-heterozygotes showed lower survival rates when infected with *E. coli* (non-infected survival of 77.2±3.7% compared with infected survival of 47.6±3.0%) or *M. luteus* (non-infected survival of 90.1±1.9% compared with infected survival of 32.9±9.1%). Therefore, both *14-3-3*ε mutant alleles rendered *Drosophila* susceptible to death following bacterial infection.

Immune-response tissues differentiate normally in *14-3-3*ε mutant larvae

To exclude the possibility of abnormal fat body and haemocyte development in *14-3-3*ε mutants, we performed morphological analysis on these tissues. There were no obvious defects in the size, overall tissue morphology or apoptosis of the fat body or

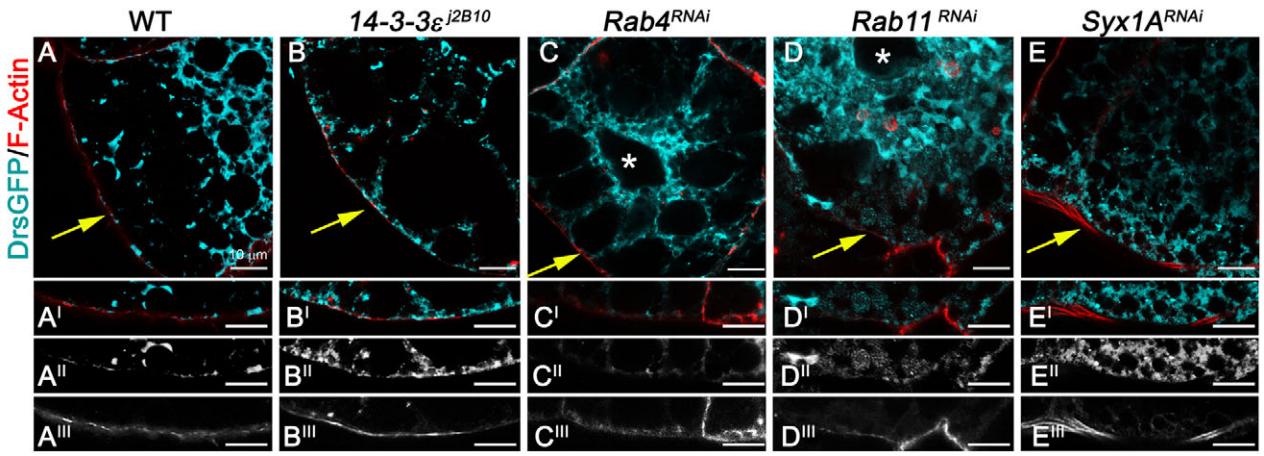

Fig. 2. Loss of 14-3-3ε leads to accumulation of Drs–GFP at the plasma membrane of fat body cells. (**A**–**E**) Confocal micrographs showing the intracellular distribution of Drs–GFP (turquoise) in fat body cells. Arrows in depict the plasma membrane outlined by phalloidin–Alexa-Fluor-568 staining for F-Actin (red). Asterisks indicate the positions of the nuclei. (**A^{I}–E^{III}**) Peri-membranous areas of the fat body cells showing the distribution of Drs–GFP (turquoise in A^{I}–E^{I}; greyscale in A^{II}–E^{II}) relative to the F-Actin outlined plasma membrane (red in A^{I}–E^{I}; greyscale in A^{III}–E^{III}). Representative larval fat body cells were from the following genotypes: (A–A^{III}) wild type (WT); (B–B^{III}) *14-3-3ε^{j2B10}* homozygotes; (C–C^{III}) *CG-GAL4>UAS-Rab4^{RNAi}*; (D–D^{III}) *CG-GAL4>UAS-Rab11^{RNAi}*; and (E–E^{III}) *CG-GAL4>UAS-Syx1a^{RNAi}*. Scale bars: 10 µm.

blood cells (either haemocytes or crystal cells), from *Drosophila 14-3-3ε* mutant third-instar larvae when compared with those in wild-type controls (supplementary material Fig. S1A–F). In addition, similar amounts of Dronc caspase (Dorstyn and Kumar, 2008) cleavage products were detected on western blots from both mutant and wild-type fat body tissue lysates, indicating similar levels of apoptosis (supplementary material Fig. S1G). One of the characteristics of a fully differentiated fat body is the accumulation of lipids as lipid droplets (Beller et al., 2006). There was no apparent change in content and distribution of neutral lipids or cholesterol observed in *14-3-3ε* mutant flies (detected by Nile Red or Fillipin staining, respectively; supplementary material Fig. S1A–D). In addition, there were no obvious differences in the number of blood cells (haemocytes and crystal cells) in *14-3-3ε* mutants when compared with the wild-type controls (supplementary material Fig. S1E,F). The crystal cells had a similar distribution along the mutant and wild-type larval bodies (supplementary material Fig. S1E,F). Finally, haemocytes and fat body cells of *14-3-3ε* mutants expressed a normal set of immune-system-related genes [*Croquemort* (*Cqr*), *PGRP-LC* and *PGRP-SA*] and at similar levels to those in wild-type controls [as detected by RT-PCR and quantitative real-time PCR (qRT-PCR) in RNA samples from the relevant tissues; supplementary material Fig. S1H–J]. This indicated that the *14-3-3ε* mutants possessed properly and terminally differentiated immune response tissues.

Defective secretion of Drs from immune-response cells in *14-3-3ε* mutants

The susceptibility of *14-3-3ε* mutant larvae to oral infection suggested a specific defect in immune function. To study the underlying molecular cause, we tested whether transcriptional activation of AMPs occurred in the fat bodies of *14-3-3ε* mutant third-instar larvae. We assessed the amount of *Drs* and *Dpt* transcripts as a measure of activating the Toll (sensing Gram-positive bacteria) and IMD (sensing Gram-negative bacteria) signalling pathways. This was assessed by qRT-PCR, in wild-type and mutant fat body tissues, either with or without oral bacterial challenge (Gram-positive *M. luteus* or Gram-negative *E. coli*, 4 hours at 25°C). No differences were observed in the basal levels of the AMP transcriptional activation between wild-type and *14-3-3ε* mutant samples (supplementary material Fig. S2). Under the mild oral immune challenge conditions, both wild-type and *14-3-3ε* mutant fat body cells exhibited a similar trend of transcriptional upregulation for both *Drs* and *Dpt* when compared with the fat body cells from non-challenged larvae (supplementary material Fig. S2). These results indicate that the lack of 14-3-3ε protein does not impair the transcriptional activation of Toll and IMD pathways.

To demonstrate that AMP transcripts were translated in *14-3-3ε* mutants, we employed a previously characterised *Drs-GFP* transgene, the expression of which is controlled by the endogenous *Drs* promoter and is therefore responsive to the activation of the Toll pathway (Ferrandon et al., 1998). This Drs–GFP fusion protein retains the native secretion signal peptide sequence and has been shown to be secreted into the haemolymph (Ferrandon et al., 1998). The detection of Drs–GFP signal in fat body and haemocytes cells from *14-3-3ε* mutant larvae, following oral bacterial stimulation with *M. luteus*, indicated no defect in the translation of this secretory AMP (Fig. 2A,B; Fig. 3A,B; Fig. 4B,C).

To investigate whether Drs–GFP was secreted into the haemolymph we quantified the intensity of GFP signal in haemolymph samples from the wild-type and *14-3-3ε* mutants (Fig. 3C,D). This analysis showed a 34.5% reduction in the intensity of Drs–GFP haemolymph signal in *14-3-3ε* mutants when compared with that in wild-type, indicating reduced Drs–GFP secretion in the mutants. This reduced secretion coincided with abnormal accumulation of Drs–GFP-positive vesicles near the plasma membrane of *14-3-3ε* mutant cells, when compared with that in wild-type controls (Fig. 2A,B; Fig. 3A,B; Fig. 4B,C). This suggests that there is a potential problem in the AMP delivery process, possibly either at the recycling endosome or at the plasma membrane.

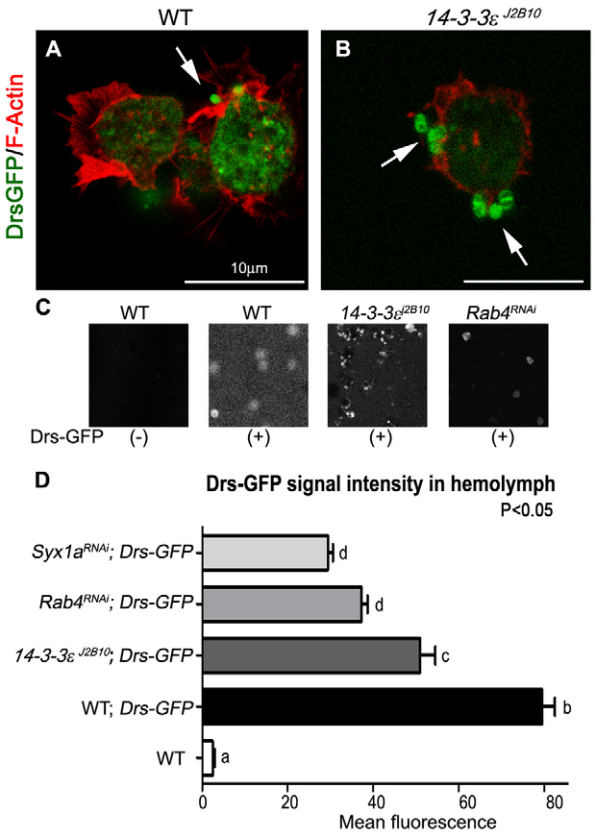

Fig. 3. Loss of 14-3-3ε leads to reduced Drs–GFP in the haemolymph and Drs–GFP accumulation near the plasma membrane of haemocytes. (**A**,**B**) Confocal micrographs showing the intracellular distribution of Drs–GFP (green) relative to the plasma membrane outlined by phalloidin–Alexa-Fluor-568 staining for F-Actin (red). (A) Wild-type (WT) and (B) *14-3-3ε*^{j2B10} homozygous haemocytes. Arrows show Drs–GFP-positive punctae near the haemocyte plasma membrane. (**C**) Representative confocal micrographs showing Drs–GFP signal in the haemolymph collected from third-instar larvae of the following genotypes: wild-type without Drs–GFP transgene (negative control for autofluorescence) and with the Drs–GFP transgene (positive control); Drs-GFP transgene in *14-3-3ε*^{j2B10} homozygotes and an RNAi-silenced Rab4 genetic background (*CG-GAL4>UAS-Rab4*^{RNAi}). (**D**) Histogram showing the quantified levels of Drs–GFP signal in the designated genotypes. Results are means±s.e.m. One-way ANOVA and Tukey's multiple comparison test showed significant differences (P<0.05) between the means in genotypes, depicted by different letters on the bars. Scale bars: 10 μm.

Recycling endosomes and Drs delivery

We investigated the intracellular trafficking of Drs and its relationship to Rab11- and Rab4-positive recycling endosomes, which are involved in cargo delivery to the plasma membrane (Clarke et al., 2006; Sato et al., 2008; Satoh et al., 2005; Ward et al., 2005). In wild-type cells, Drs–GFP was included into Rab4–mRFP-positive (Fig. 4A) and Rab11-positive vesicles (Fig. 4B). The RNA interference (RNAi)-mediated silencing of Rab4 resulted in the accumulation of Drs–GFP, mainly within the perinuclear region of fat body cells (compare Fig. 2C with 2A), and correlated with a 53% reduction in the intensity of Drs–GFP signal in the haemolymph, indicating reduced secretion (Fig. 3C,D). The RNAi silencing of Rab11 also led to the perinuclear accumulation of Drs–GFP in most fat body cells (Fig. 2D), but this did affect Drs–GFP expression, as well as morphology and possibly integrity in some cells. On the basis of this irregular Drs–GFP expression in fat body cells, the *Rab11*^{RNAi} haemolymph sample was omitted from the analysis. These results suggest a role for Rab4 and Rab11 in the anterograde traffic of Drs–GFP from the perinuclear region towards the plasma membrane. There did not appear to be any disruption to recycling endosome compartments in *14-3-3ε* mutants because in both wild-type and *14-3-3ε* mutants there was a similar distribution and number of the Rab11–GFP and Rab4–mRFP double-positive vesicles (Fig. 4D,E).

Drs and small Rab11-positive vesicles are concentrated near the plasma membrane in cells from *14-3-3ε* mutants

Having shown the abnormal distribution and secretion of Drs in *14-3-3ε* mutants (Fig. 2A,B; Fig. 3A,B), we investigated further the Rab11-positive vesicles that are involved in vesicle targeting to the plasma membrane. In the wild-type and *14-3-3ε* mutant fat body cells, Drs–GFP was found inside small Rab11-positive vesicles that were located near the plasma membrane (detected by an anti-Rab11 antibody; Fig. 4B,C). There was, however, an accumulation of small Rab11-positive vesicles at the plasma membrane of *14-3-3ε* mutant fat body cells, but not in the wild-type fat body, as established by anti-Rab11 antibody staining (Fig. 5A,C) and Rab11–GFP fusion protein expression (using the fat-body- and haemocyte-specific driver *CG-GAL4*; Fig. 5B,D). Ultrastructural analysis by electron microscopy demonstrated some vesicles near the plasma membrane of wild-type fat body cells, but there were more vesicles in *14-3-3ε* mutant cells (supplementary material Fig. S3). Quantitative analysis showed a significant increase (P<0.05) in the number of Rab11-positive vesicles in *14-3-3ε* mutants when compared with that in wild-type controls (Fig. 5E).

To confirm the specificity of the Rab11-vesicular phenotype with the loss of 14-3-3ε protein, a copy of wild-type *14-3-3ε* cDNA was introduced into the *14-3-3ε* mutant background, using the fat-body- and haemocyte-restricted expression of *CG-GAL4>UAS-14-3-3ε* transgenes ('rescue assay'). The number of Rab11–GFP-positive vesicles in the rescue assay did not differ significantly from that in the wild type (Fig. 5E, see *UAS-14-3-3ε; 14-3-3ε*^{j2B10}). In addition, the fat-body-restricted RNAi silencing of *14-3-3ε* by expression of *CG-GAL4>UAS-14-3-3ε*^{RNAi} transgenes produced a phenotype similar to that of *14-3-3ε* mutants (Fig. 5E). Together, this indicates that the phenotype is caused by the loss of 14-3-3ε in haemocytes and fat body tissues. The role of the 14-3-3ζ homologue was also investigated in wild-type and *14-3-3ε* mutant backgrounds using *CG-GAL4>UAS-14-3-3ζ*^{RNAi} transgenic expression. The RNAi silencing of *14-3-3ζ* also increased the number of Rab11–GFP-positive vesicles located at the plasma membrane when compared with that in wild-type controls (Fig. 5E, *UAS-14-3-3ζ*^{RNAi}). However, the number of plasma membrane associated vesicles was significantly higher in *14-3-3ε* mutants when compared with that upon *14-3-3ζ* RNAi silencing (Fig. 5E). Moreover, introduction of the *14-3-3ζ*^{RNAi} transgene into the *14-3-3ε* mutants did not significantly increase the number of membrane-localised Rab11–GFP vesicles when compared with that in the *14-3-3ε* mutant alone (Fig. 5E, *UAS-14-3-3ζ*^{RNAi}*; 14-3-3ε*^{j2B10}). There was a similar number of vesicles accumulating at the plasma membrane in *14-3-3ζ*^{RNAi} cells when compared with that in the 'rescued' *14-3-3ε* mutants. These results suggest that there is a substantial role for *Drosophila* 14-3-3ε in the control of Rab11-positive vesicles in the fat body and a lesser functional role for 14-3-3ζ.

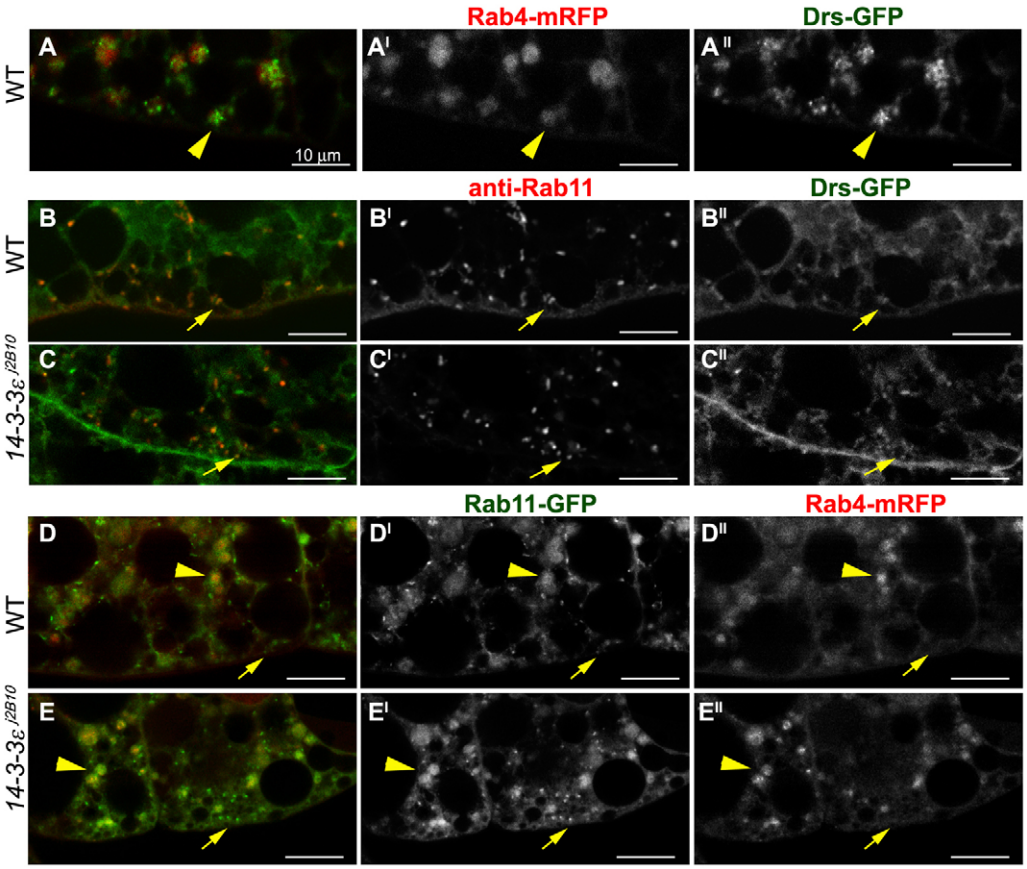

Fig. 4. Drs–GFP colocalised with Rab4- and Rab11-positive vesicles in fat body cells. (**A**–**A^{II}**) Micrographs of confocal cross-sections though the cells showing the colocalisation of Rab4–mRFP-positive vesicles (red in A, greyscale in A^{I}) with Drs–GFP (green in A, greyscale in A^{II}) in wild-type (WT) cells. Arrowheads denote the double Rab4–mRFP- and Drs–GFP-positive vesicles. (**B**–**C^{II}**) Micrographs of confocal cross-sections though the cells showing the colocalisation of Rab11-positive vesicles (detected with an anti-Rab11 antibody; red in B and C, greyscale in B^{I} and C^{I}) with Drs–GFP (green in B and C; greyscale in B^{II} and C^{II}). The fat body cell was from (B–B^{II}) wild-type and (C–C^{II}) *14-3-3ε^{j2B10}* homozygotes. Arrows denote the double Rab11- and Drs–GFP-positive vesicles. (**D**–**E^{II}**) Micrographs of confocal cross-sections though the cells showing the colocalisation of Rab11-GFP (green in D and E; greyscale in D^{I} and E^{I}) with Rab4-mRFP-positive vesicles (red in D and E; greyscale in D^{II} and E^{II}). The fat body cells were from (D–D^{II}) wild-type and (E-E^{II}) *14-3-3ε^{j2B10}* homozygotes. Arrowheads in denote the double Rab11–GFP- and Rab4–mRFP-positive vesicles, arrows denote the Rab11-GFP-only positive vesicles. The appearance of large Rab11–GFP-positive and Rab4–RFP-positive vesicles might result from the ectopic expression of the respective transgenes. Scale bars: 10 μm.

Next, we examined the distribution of 14-3-3ε in relation to the Rab11-positive vesicles. In wild-type *Drosophila*, 14-3-3ε was observed as punctuate staining in close proximity to Rab11–GFP-positive vesicles (Fig. 6A,B). In addition, 14-3-3ε occasionally colocalised with the vesicular Syntaxin proteins, especially near the plasma membrane (Fig. 6C). The phenotype of RNAi silencing of *Drosophila* Syntaxin 1a (Syx1a) closely mimicked the phenotype seen for the *14-3-3ε* mutants, namely the accumulation of Drs–GFP, Rab11-positive vesicles near the plasma membrane and a 63% reduction in the Drs–GFP signal in the haemolymph (Fig. 2E; Fig. 3D; Fig. 5E). Given the function of Syx1a as a component of the exocytic machinery (Gladycheva et al., 2007; Murray et al., 2005; Pagan et al., 2003), the similarity between the *14-3-3ε* and the *Syx1a^{RNAi}* phenotypes suggests that 14-3-3ε has a role in regulating exocytosis.

Discussion

Members of the evolutionary conserved 14-3-3 family of proteins have previously been implicated in the regulation of various signalling pathways for cell survival, proliferation and development (Acevedo et al., 2007; Benton et al., 2002; Broadie et al., 1997; Chen et al., 2003; Darling et al., 2005; Grammenoudi et al., 2008; Guthridge et al., 2004; Guthridge et al., 2006; Karam et al., 2010; Li et al., 1997; Skoulakis and Davis, 1996; Skoulakis and Davis, 1998; Su et al., 2001; Thomas et al., 2005). However, the specific action of these proteins has been difficult to define, mainly because of the functional redundancy between 14-3-3 homologues in vertebrates. In *Drosophila* there are only two homologues, 14-3-3ε and 14-3-3ζ, and therefore *Drosophila* represent an appealing in vivo system in which to study the role of distinct 14-3-3 isoforms. Despite 14-3-3ε and 14-3-3ζ having a high degree of sequence identity (62%) (Skoulakis and Davis, 1998), functional differences have begun to emerge. Thus, whereas *14-3-3ζ* protein-null mutants are embryonic lethal, suggesting a crucial role in embryonic development, *14-3-3ε* mutants survive to adulthood and exhibit no obvious morphological or functional defects, except for sterility (Acevedo et al., 2007; Benton et al., 2002; Messaritou et al., 2010). These results raised the possibility that *14-3-3ε* was required for some tissue-specific functions.

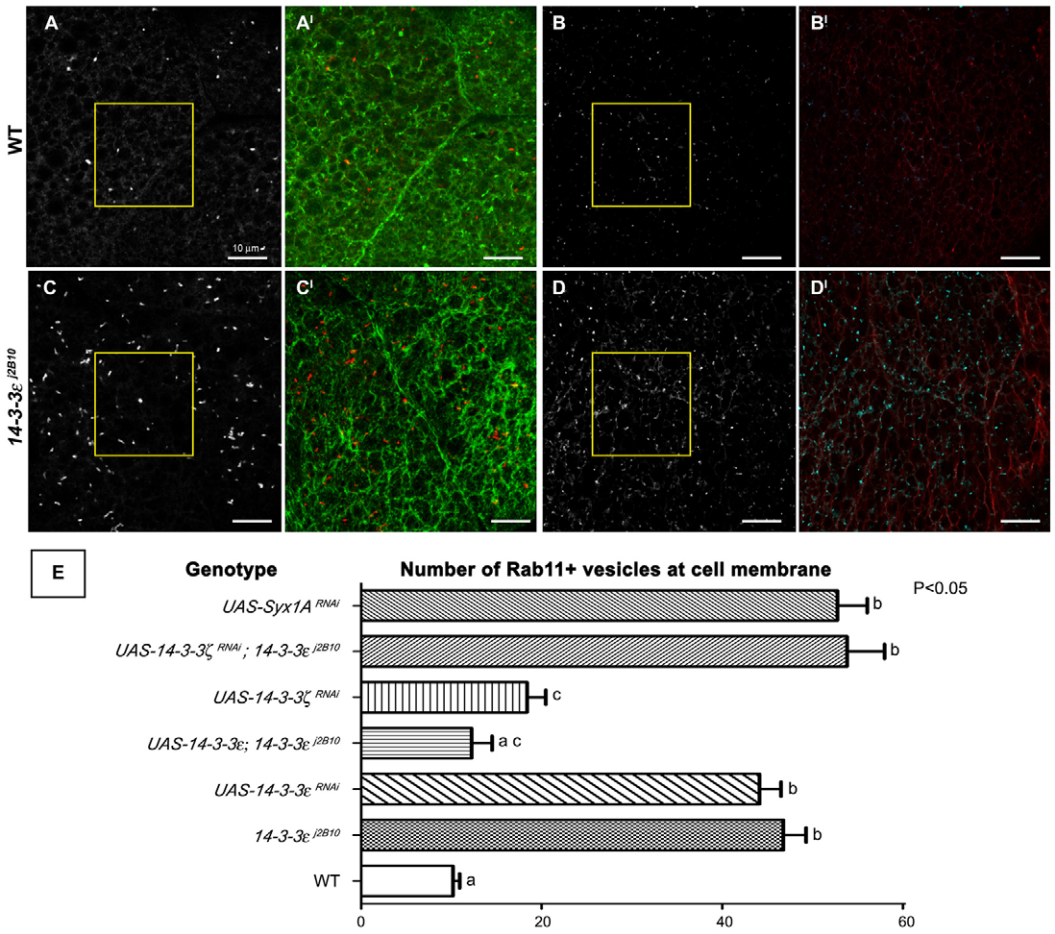

Fig. 5. *14-3-3*ε mutants showed accumulation of Rab11-positive vesicles at the plasma membrane of fat body cells. (A–D^{I}) Confocal micrographs showing the Rab11-positive vesicles at the apical surface of the fat body cells, with the membrane outlined by phalloidin–Alexa-Fluor-568 staining for F-Actin (green in A^{I} and C^{I}; red in B^{I} and D^{I}). The Rab11-positive vesicles was detected with an anti-Rab11 antibody (greyscale in A and C; red in A^{I} and C^{I}) or with Rab11–GFP expression induced by *CG-GAL4>UAS-Rab11-GFP* transgenes (greyscale in B and D; turquoise in B^{I} and D^{I}). Representative fat body cells are from (A–B^{I}) wild-type (WT) and (C–D^{I}) *14-3-3*ε^{j2B10} homozygous third-instar larvae. Scale bars: 10 μm. (**E**) Histogram showing the mean number (+s.e.m.) of Rab11-positive vesicles scored in two squares (19.6 μm ×19.6 μm, shown in yellow in A and B) per larvae, in seven to ten third-instar larvae per designated genotype (UAS lines were driven by *CG-GAL4*). One-way ANOVA showed significant differences between means (P<0.05). Tukey's multiple comparison test showed significant differences between the means in the genotypes, when is depicted by different letters on the right-hand side of the bars (P<0.05); the genotypes with common letters showed no significant difference between the means.

Here, we have shown that *14-3-3*ε mutant larvae had significantly reduced survival rates when orally infected with bacteria, indicating an important role for 14-3-3ε in innate immunity. Developmental, differentiation and metabolic defects were excluded as the cause of the observed phenotype in *Drosophila 14-3-3*ε mutants by demonstrating similar survival rates for the respective developmental stages in wild-type *Drosophila*. Thus, there was no obvious change in the level of apoptosis in these tissues, with equivalent Dronc caspase cleavage in *14-3-3*ε mutant and wild-type *Drosophila*. Even under conditions of immune challenge there were no identifiable defects in the morphology or maturity of immune response tissues, including the haemocytes and fat body.

Studies over the past decade have provided some understanding as to how the two evolutionary conserved pathways of Toll/IL-1 receptor and IMD–TNFα/Eiger (Govind and Nehm, 2004) control innate immunity, in response to a specific microbial challenge. For example, immune challenge leads to a rapid and prolific transcriptional activation of AMPs (Imler and Bulet, 2005). In immune-challenged *14-3-3*ε mutants, the key genes of the Toll and IMD immune response pathways were activated, resulting in *Drs* and *Dpt* AMP expression. There was therefore a normal transcriptional initiation of the immune response in *Drosophila 14-3-3*ε mutants. In the absence of any immune challenge, AMPs might still have been activated in a transcriptional factor FOXO/Forkhead-dependent-manner (Becker et al., 2010), because 14-3-3 proteins are known to bind phosphorylated FOXO, which can prevent nuclear translocation and transcriptional activation of FOXO target genes (van der Heide et al., 2004). However, our analysis of *14-3-3*ε mutants in the absence of microbial challenge showed no abnormal AMP activation, suggesting that 14-3-3ε did not have a role in AMP transcriptional regulation.

In *Drosophila*, AMPs function as immune mediators, and the regulation of their secretion is a crucial step in organism self-defence (Imler and Bulet, 2005). Here, *14-3-3*ε mutants demonstrated impaired secretion of the antimicrobial peptide Drs, into the haemolymph and concomitant accumulation of Drs-positive

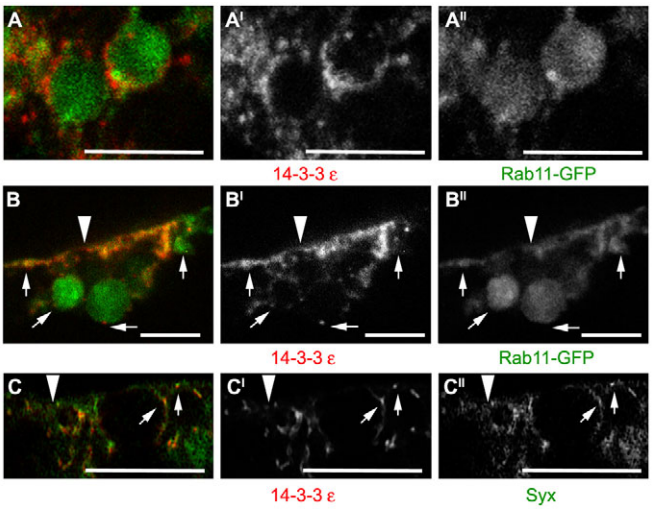

Fig. 6. Localisation of 14-3-3ε in relation to Rab11 compartments for wild-type fat body cells. (**A**–**B^{II}**) Confocal micrographs showing localisation of 14-3-3ε detected with anti-14-3-3ε antibody (red in A and B; greyscale in A^{I} and B^{I}) in wild-type fat bodies labelled with Rab11–GFP fusion protein from the *CG-GAL4>UAS-Rab11-GFP* transgenic marker (green in A and B; grayscale A^{II} and B^{II}). (**C**–**C^{II}**) Confocal micrographs showing localisation of 14-3-3ε detected with anti-14-3-3ε antibody (red in C; greyscale in C^{I}) in relation to the general distribution of Syntaxins detected by anti-Syntaxin antibody 8C3 (green in C; greyscale in C^{II}). The arrowheads in B–C^{II} denote the position of the plasma membrane. Arrows in B–B^{II} depict the 14-3-3ε-positive signal in close proximity to Rab11–GFP-positive vesicles. Arrows in C–C^{II} point towards the colocalisation of 14-3-3ε-positive and Syntaxin-positive signals. Data is representative of at least ten independent replicates. Scale bars: 5 µm.

vesicles near the plasma membrane of fat body cells and haemocytes. In *Drosophila 14-3-3ε* mutants, this reduction in AMP secretion would have an impact upon the capacity to kill bacteria and might explain the susceptibility to death following bacterial challenge. However, we cannot exclude the possibility that the defective accumulation of the Rab11-GFP-positive vesicles in *14-3-3ε* mutants impairs secretion of either other immune mediators or soluble proteins that impact upon larval viability.

A current model for cargo delivery to the plasma membrane implicates Rab4 and Rab11 in coordinated protein trafficking to the plasma membrane (Li et al., 2008; Ward et al., 2005). According to this model, proteins are sorted into Rab4- and Rab11-positive compartments for additional sorting, and then are delivered to the plasma membrane in Rab11-positive vesicles. This function of Rab4 and Rab11 appears to be conserved in *Drosophila* as there were two vesicular populations in fat body cells, large Rab4- and Rab11-positve intracellular vesicles and smaller Rab11-positive vesicles. Drs resided in both of these types of vesicles, but it is probable that it is the smaller Rab11-only positive vesicles that are involved in the delivery of cargo to the plasma membrane. A role for *Drosophila* Rab4 and Rab11 in cargo delivery towards the plasma membrane is supported by the finding that RNAi silencing of these GTPases led to Drs accumulation, mainly in the perinuclear region. By contrast, the loss of the 14-3-3ε protein resulted in Drs and small Rab11-positive vesicle accumulating near the plasma membrane of fat body cells.

In wild-type controls, 14-3-3ε was found directly adjacent to Rab11-positive vesicles. Given the established function of 14-3-3ε as a mediator of protein–protein interactions (Mackintosh, 2004), 14-3-3ε might function in the assembly of the protein complex essential for the final delivery of Rab11 vesicles to and/or fusion with the plasma membrane. The capture and docking of exocytic vesicles at target membranes involves tethering factors and members of the SNARE family of proteins. In cells of the immune system, SNARE-mediated vesicle-to-plasma-membrane fusion is crucial for the release of stored inflammatory mediators and immune effectors from secretory granules (Lieu et al., 2008; Manderson et al., 2007; Murray et al., 2005; Pagan et al., 2003). The specificity of membrane fusion events is regulated by the composition of protein complexes containing different SNAREs (Syntaxins, SNAPs, etc.), including, for instance, Syx1a (Gladycheva et al., 2007). There was a marked similarity in the accumulation of Drs- and Rab11-positive vesicles observed in the *Drosophila 14-3-3ε* mutants and upon *Syx1a* RNAi silencing. Notably, this effect closely resembles the effect of the null mutants for the vertebrate syntaxin-binding protein 1 (STXBP1; also known as UNC18-1), the crucial effector of exocytosis of cytotoxic granules in T lymphocytes (Ménasché et al., 2008; Tomas et al., 2008; Verhage and Sorensen, 2008). A genome-wide yeast two-hybrid screen has demonstrated interaction of *Drosophila* 14-3-3ε and Syx1a (Giot et al., 2003). In our study, there was some colocalisation between Syntaxin and 14-3-3ε in fat body cells. These findings suggest a potential regulatory role for 14-3-3ε in Rab11-positive vesicle delivery and/or release, which might involve Syx1a.

There is a possibility that the role of 14-3-3ε in exocytosis is mediated by modulation of the cytoskeleton because 14-3-3 proteins have been previously implicated in the regulation of microtubule and actin polymerisation (Birkenfeld et al., 2003; Sadik et al., 2009; Timm et al., 2006). In *14-3-3ε* mutant fat body cells, both Drs- and Rab11-positive vesicles are delivered into close proximity to the plasma membrane, indicating that the microtubule-dependent long-range trafficking is not affected (Dollar et al., 2002; Rodriguez-Boulan et al., 2005; Sadik et al., 2009; Sonnichsen et al., 2000; Vega and Hsu, 2001). The final delivery of exocytic vesicles and fusion to the plasma membrane depends on the polymerisation state of actin filaments and the integrity of actin-associated protein complexes (Jacobs et al., 2009; Yu and Bement, 2007). In yeast, the overexpression of the dominant-negative allele of one of the 14-3-3 orthologues, Bmh2p, has been shown to cause some defects in actin organisation and vesicle targeting (Roth et al., 1999). Despite the fact that there was no obvious disruption to F-actin organisation in our *Drosophila* 14-3-3ε mutants, 14-3-3-mediated regulation of actin (e.g. through the actin-depolimerising factor cofilin) (Birkenfeld et al., 2003) might contribute to some actin cytoskeleton modifications at or near the plasma membrane. Thus, we cannot exclude the possibility that undetectable rearrangements have prevented the final docking of the Rab11 vesicles. Alternatively, a defect in actin dynamics at the plasma membrane could have interfered with the retrieval of these vesicles by compensatory endocytosis (Schafer, 2003). Further investigation will be required to discern the precise molecular basis of the defect in *14-3-3ε* mutants.

In vertebrates, 14-3-3 proteins have been implicated in the targeting of specific cargo proteins to the plasma membrane, including delivery of the K^+ channel proteins KCNK3 and KNCK9, the ATP-sensitive K^+ channel Kir2.1 and the major histocompatibility complex (MHC) class II invariant chain (Michelsen et al., 2006; Mrowiec and Schwappach, 2006; Shikano et al., 2005). The present study suggests that *Drosophila* 14-3-3ε

acts as a general regulator of exocytic machinery. The previously described memory dysfunction in *Drosophila 14-3-3ζ* mutants (Philip et al., 2001; Skoulakis and Davis, 1998) would support this conclusion, particularly as neurotransmitter release at neurological synapses is highly dependent on exocytosis (Menager et al., 2007).

In summary, our results show that 14-3-3ε has a crucial role in innate immunity and is essential for *Drosophila* viability under bacterial challenge. This crucial function in innate immunity is probably linked to the role of 14-3-3ε as a regulator of AMP secretion. However, by regulating Rab11-dependent exocytosis, 14-3-3ε might be implicated in other secretion processes. The discovery that 14-3-3ε regulates immune secretion in *Drosophila* raises the question of which other proteins interact with 14-3-3ε during this process. Recent proteome analysis identified some potential 14-3-3-binding partners as components of the vesicle trafficking machinery in human cells [LYST (also known as Chediak-Higashi), SEC23, VPS33B, clathrin heavy chain, Rab GTPases, and their GTPase-activating protein and guanine-exchange-factor (i.e. GAP and GEF) regulators] (Jin et al., 2004; Pozuelo Rubio et al., 2004; Tchernev et al., 2002) and in *Drosophila* phagosomes (Stuart et al., 2007). The scene is now set to define the molecular partners of 14-3-3ε and the precise function of this protein complex in the human immune response. The specific involvement of 14-3-3 proteins in human immune-related diseases is still to be fully explored, but the in vitro interaction of mammalian 14-3-3 proteins with LYST and Sec1/Vps33B, the proteins respectively linked to Chédiak–Higashi and Hermansky–Pudlak syndromes, suggests the need for a genetic screening for 14-3-3 mutations.

Materials and Methods

Fly stocks

Unless specified, fly stocks were obtained from the Bloomington *Drosophila* Stock Center (Indiana University, IN). For targeted expression of genes of interest, the yeast GAL4-UAS system was used (Brand and Perrimon, 1993). Fat-body- and haemocyte-specific expression of transgenes from the UAS was driven by *CG-GAL4* (Asha et al., 2003). *UAS-14-3-3ζ*^{RNAi} and *UAS-Syx1a*^{RNAi} transgenes were obtained from the Vienna *Drosophila* RNAi Centre (Dietzl et al., 2007). Transgenic stocks *UAS-Rab4-mRFP, UAS-Rab4*^{S22N} and *UAS-Rab11-GFP* were obtained from Markos González-Gaitán (University of Geneva, Geneva, Switzerland) (Entchev et al., 2000; Wucherpfennig et al., 2003) and Donald F. Ready (Purdue University, West Lafayette, IN) (Satoh et al., 2005). *Drs-GFP* was a kind gift from Dominique Ferrandon (Equipe Fondation Recherche Médicale, Strasbourg, France) (Ferrandon, 2007). The *UAS-14-3-3ε, 14-3-3ζ*^{leo1188} and the two *14-3-3ε* alleles used in this study, the protein-null allele *14-3-3ε*^{j2B10} (a P/SUPor-P/ insertion at position +2 of the 5′-UTR) and the point mutant *14-3-3ε*^{E183K}, were as previously described (Acevedo et al., 2007).

Natural bacterial infection

The early third-instar larvae were placed in petri dishes with a grape juice agar layer, covered with 100 µl of a bacterial suspension (OD_{600} ~200) in 5% sucrose (*E. coli* DH5α for 11 hours at 25°C; *M. luteus* for 7 hours at 29°C). The control larvae were reared on 5% sucrose for an equal length of time. The experiments were repeated seven to ten times to achieve a sufficient sample size. To assess the survival rates following infection, larvae and pupae were scored as dead and alive at 6-hour intervals. For each group, n=500–700 individual larvae were scored. Comparisons between individual data points were performed using Student's t-test. Results were considered significant if $P<0.05$. For gene expression qRT-PCR and Drs–GFP expression analysis, bacterial infection assays were performed at 25°C (to avoid temperature stress) for 4 hours to collect the fat body samples prior to larval death.

Gene expression analyses

For RT and qRT-PCR analysis, RNA from fat bodies from ten third-instar larvae were prepared using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. cDNA was generated using the OmniScript kit protocol (Qiagen). Go Taq Green Master Mix (Promega) was used for RT-PCR, and SYBR Green qPCR kit (Qiagen) for qRT-PCR. The following primers were used for RT-PCR and qRT-PCR: *Toll* (CG5490) forward, 5′-ACATCTGCCTTTCTCCAACC-3′, and reverse, 5′-TGTTGTCCAAATGGGTTCAT-3′; *14-3-3ε* (CG31196) forward, 5′-AAGTCCGT-CATTAGGCCATC-3′, and reverse, 5′-TGTTCGATCGAGGTGATGAT-3′; *PGRP-SA* (CG11709) forward, 5′-CCTTCGTTGGGACTCCACTA-3′, and reverse 5′-CGT-GTGATGGATGACCACAT-3′; *PGRP-LC* (CG4432) forward, 5′-GGCAGTTC-CAATCGAAATCG-3′, and reverse, 5′-CGCTGATACGCTTGGATTCC-3′; *rp49* (CG7939) forward, 5′-CAGGCCCAAGATCGTGAA-3′, and reverse, 5′-CAAATGT-GTATTCCGACC-3′; *Drosomycin* (CG10810) forward, 5′-GTACTTGTTCGCC-CTCTTCG-3′, and reverse, 5′-ATTTAGCATCCTTCGCACCA-3′; *Diptericin* (CG12763) forward, 5′-GAGATGCAGTTCACCATTG-3′, and reverse, 5′-TTTCCAGCTCGGTTCTGAGT-3′; *Croquemort* (CG4280), forward 5′-CCTACG-TATCCACCATGTGCT-3′, and reverse 5′-GGGTAAGGCCATCCTCTACA-3′. The in vivo efficiency of *UAS-RNAi* transgenes was assessed by qRT-PCR using the following primers: *Syntaxin1a* (CG31136) forward, 5′-TAAAGCCCGACGAAA-GAAGA-3′, and reverse, 5′-GTGGAGAGGTGTGTGTGTGG-3′; *14-3-3ζ* (CG17870) forward, 5′-GTCACAGAGACTGGCGTTGA-3′, and reverse, 5′-GGAG-GAGATGACACGCCACGA-3′. For western blotting analysis, protein extracts were pulled from ten third-instar larvae of wild-type, *14-3-3ε*$^{j2B10/j2B10}$ and *14-3-3ε*$^{j2B10/E183K}$ mutants and from 100 homozygous *14-3-3ζ*$^{leo1188/1188}$ embryos and were analysed according to the protocol described previously (Gronke et al., 2003).

Histology and immunofluorescence analysis

Fillipin and Nile Red (both Invitrogen) staining was conducted as previously described (Gronke et al., 2007). Antibodies used for immunofluorescence were: rabbit anti-14-3-3ε antibody (two independent batches, A8 and A10, from two inoculated rabbits, a gift from Cheng-Ting Chien, Institute of Molecular Biology, Taipei, Taiwan) (Tien et al., 1999), rabbit anti-14-3-3ζ/Leo antibody (Kockel et al., 1997), two independent rabbit anti-Rab11 antibodies [obtained from Robert S. Cohen (University of Kansas, Lawrence, KS) (Dollar et al., 2002) and from Akira Nakamura (Tanaka and Nakamura, 2008)], mouse monoclonal anti-Syntaxin antibody 8C3 (Developmental Studies Hybridoma Bank, Iowa, USA) (Fujita et al., 1982), anti-IgG secondary antibodies conjugated with biotin, Cy3, Cy5 or SA-Cy3 (Jackson ImmunoResearch Laboratories), or Alexa-Fluor-488, and Phalloidin–Alexa-Fluor-568 (Molecular Probes). Fixed and stained tissues were viewed with a Zeiss Axiophot microscope (for transmitted light), or viewed with either an Olympus Provis AX70 epifluorescence microscope or a confocal microscope equipped with a BioRad MRC1000 scanhead and a krypton/argon laser (for fluorescence). Confocal micrographs represent 1-µm optical sections.

Tissues for transmission electron microscopy (TEM) analysis were prepared according as described previously (Kohler et al., 2009) and viewed with a Philips Technai 12 transmission electron microscope (The Centre for Microscopy and Microanalysis, The University of Queensland, Australia).

To test for secretion of Drs–GFP into the haemolymph, we bacterially challenged third-instar larvae of wild-type or *14-3-3ε* mutant genotypes carrying a *Drs-GFP* transgene. After 4 hours of challenge, the level of GFP fluorescence in larvae was checked under the fluorescent dissection microscope (Olympus, Japan) and if detected, haemolymph was collected from each larvae by gently puncturing with a 26-gauge needle to let the haemolymph bleed onto a coverslip. The exposure to air induces a rapid and extensive coagulation of insect haemolymph, entrapping haemocytes within the clot (Bidla et al., 2005; Karlsson et al., 2004; Scherfer et al., 2004). The distinct composition of the insect clot (Theopold et al., 2002) makes it insensitive to detergent and vertebrate anti-coagulation reagents. To overcome this problem, the quantitative analysis of the Drs-GFP signal in haemolymph was carried out before the formation of an insoluble clot: immediately after bleeding, the drop of haemolymph was covered with mineral oil and the intensity of fluorescent signal was quantified in the haemocyte-free regions of the haemolymph in 3-µm confocal sections (BioRad MRC1000 scanhead and a krypton-argon laser). A total of 12 larvae per genotype (at least three independent regions per larvae) were analysed.

Statistical analysis

Data are presented as means±s.e.m. The difference between group means was assessed by one-way analysis of variance (ANOVA), with individual group variance assessed by a Bartlett's test. Where the level of significance was $P<0.05$, post-hoc tests were performed using a Tukey's multiple comparison test (GraphPad Prism software).

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Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/124/13/2165/DC1

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