The full-of-bacteria gene is required for phagosome maturation during immune defense in Drosophila

Mohammed Ali Akbar,1 Charles Tracy,1 Walter H.A. Kahr,3 and Helmut Krämer1,2

1Department of Neuroscience and 2Department of Cell Biology, University of Texas Southwestern Medical Center, Dallas, TX 75390
3Division of Haematology/Oncology, Program in Cell Biology, Department of Pediatrics, The Hospital for Sick Children, University of Toronto, Toronto, Ontario M5G 1X8, Canada

Arthrogryposis, renal dysfunction, and cholestasis (ARC) syndrome is a fatal recessive disorder caused by mutations in the VPS33B or VPS16B genes. Both encode homologues of the Vps33p and Vps16p subunits of the HOPS complex necessary for fusions of vacuoles in yeast. Here, we describe a mutation in the full-of-bacteria (fob) gene, which encodes Drosophila Vps16B. Flies null for fob are homozygous viable and fertile. They exhibit, however, a defect in their immune defense that renders them hypersensitive to infections with nonpathogenic bacteria. fob hemocytes (fly macrophages) engulf bacteria but fail to digest them. Phagosomes undergo early steps of maturation and transition to a Rab7-positive stage, but do not mature to fully acidified phagolysosomes. This reflects a specific requirement of fob in the fusion of phagosomes with late endosomes/lysosomes. In contrast, cargo of autophagosomes as well as endosomes exhibit normal lysosomal delivery in fob cells. These findings suggest that defects in phagosome maturation may contribute to symptoms of ARC patients including recurring infections.

Introduction

Phagocytosis is an important element of the defense mechanisms against microbial invaders. Microbes are engulfed into early phagosomes by actin-driven extensions of the plasma membrane. Initial properties of phagosomes are dictated by their origination from the plasma membrane, but fusion with endosomes quickly initiates phagosomal maturation (Flannagan et al., 2009). Continued maturation of phagosomes depends on the fusion with early and late endosomal compartments, and eventually lysosomes (Kinchen and Ravichandran, 2008).

As phagosomes mature, they transition through an early stage marked by the presence of the GTPase Rab5 and its effectors (Kinchen and Ravichandran, 2008). Among them, Mon1/SAND-1 proteins aid in the conversion from Rab5- to Rab7-positive late phagosomes (Kinchen and Ravichandran, 2010), which is equivalent to their role in endosome maturation (Poteryaev et al., 2010). Rab7, subsequently, is required for phagosomes and late endosomes to fuse with lysosomes (Bucci et al., 2000; Harrison et al., 2003).

HOPS (homotypic fusion and vacuole protein sorting) is a multiprotein complex that originally was characterized in yeast for its role in vacuolar fusions (Sato et al., 2000; Seals et al., 2000). The HOPS complex acts as a tethering factor, stimulates Rab nucleotide exchange, and coordinates the interaction of SNAREs during lysosomal fusions (Nickerson et al., 2009; Wickner, 2010). In multicellular organisms, HOPS complex function is necessary for the biogenesis of lysosomes and lysosome-related organelles (Rojo et al., 2001; Sadler et al., 2005; Maldonado et al., 2006). In Drosophila, homologues of the HOPS subunits Vps18p and Vps33p are encoded by deep orange and carnation. Both genes are named for their role in the biogenesis of pigment granules in the fly eye, and, together with Vps16p, are required for lysosomal delivery of cargo from endosomes and autophagosomes (Svrivojkov et al., 1999; Srim et al., 2003; Pulipparacharuvil et al., 2005; Lindmo et al., 2006; Akbar et al., 2009). Similarly in mice, the buff mutation, because of a missense mutation in the VPS33A gene, causes abnormal pigmentation (Suzuki et al., 2003) and progressive neurodegeneration, presumably because of a defect in lysosomal delivery (Chintala et al., 2009). Furthermore, an RNAi screen in Caenorhabditis elegans...
Nonpathogenic microbes. These findings suggest that defects in phagosome maturation may contribute to symptoms of ARC patients, including their recurring infections (Gissen et al., 2006; Hershkovitz et al., 2008; Jang et al., 2009).

Results and discussion

Fob is required for normal immune defense

A $fob^1$ null allele (Fig. 1, A and B) was generated by ends-out homologous recombination (Gong and Golic, 2003). $fob^1$ flies were null for $fob$ expression but had no change in the transcription of neighboring genes (Fig. 1 C). Homozygous $fob^1$ and hemizygote ($fob^1/Df(3R)BSC547$) flies were viable, fertile, and displayed no morphological defects.

ARC patients present with recurrent infections at a high frequency (Gissen et al., 2006; Hershkovitz et al., 2008; Jang et al., 2009), thus we wondered whether microbial infections also threaten $fob^1$ flies. We compared the survival of $fob^1$ flies with and without $fob$ expression but had no change in the transcription of neighboring genes (Fig. 1 C). Homozygous $fob^1$ and hemizygote ($fob^1/Df(3R)BSC547$) flies were viable, fertile, and displayed no morphological defects.

Here, we describe a mutation in the full-of-bacteria ($fob$) gene, which encodes Drosophila Vps16B. To our surprise, flies null for $fob$ were homozygous viable and fertile. They exhibit, however, a profound defect in phagosome maturation, and as a consequence are sensitive to infections with normally nonpathogenic microbes. These findings suggest that defects in phagosome maturation may contribute to symptoms of ARC patients, including their recurring infections (Gissen et al., 2006; Hershkovitz et al., 2008; Jang et al., 2009).
Phagosome maturation requires Fob

A necessary step in the acquisition of the full degradative potential of phagosomes is their acidification, which can be monitored by imaging the fluorescence ratio of Oregon green/Texas red doubly labeled phagocytosed bacteria. After a 30–45-min chase in wild-type hemocytes, the majority of internalized bacteria appeared degraded as judged by the diffuse appearance of remaining fluorescence, but the few phagosomes that still contained well-defined bacteria had acidified to a mean pH of 5.5 ± 0.15 compared with a mean starting pH of 6.5 ± 0.15 (Fig. 3 A and Fig. S1). In contrast, fob1 phagosomes acidified only minimally, if at all: the starting mean pH of 6.6 ± 0.12 was only lowered to pH 6.3 ± 0.13 after a 30-min chase (Fig. 3 B). In accordance with this finding, electron microscopy of isolated...
of 3-phosphoinositides and subsequent recruitment of Rab5 early steps in phagosome maturation, including the generation of phosphatidylinositol 3-kinases (PI3K) and Rab5 (Stenmark et al., 1995; Vieira et al., 2003). These data indicated that effectors, are normal in fob1 mutant phagosomes. This conclusion was further supported by the presence of Avalanche (Avl), an early endosomal SNARE (Lu and Bilder, 2005), on Rbsn-5–positive phagosomes in fob1 and wild-type hemocytes (Fig. 4, A and F). This indicates that fob1 mutants have normal early endosome–phagosome fusion.

A subsequent step in phagosome maturation involves the exchange of Rab5 to Rab7, similar to their exchange observed in endosomes (Vieira et al., 2002; Rink et al., 2005). 62 ± 26% of phagosomes in fob1 hemocytes were decorated by Rab7 compared with 36 ± 14% in wild type, which indicates that Rab5-to-Rab7 conversion was not inhibited in fob1 (Fig. 4, B, B’, and F). This significantly increased presence of Rab7 on phagosomes in fob1 cells (P < 0.0001) suggests that phagosomes are stalled at this stage. This is reminiscent of the dramatic increase of Rab7 on late endosomes in car-null cells (Fig. S2 K; Akbar et al., 2009) and is consistent with Rab7 recruitment not being sufficient to induce fusion with lysosomes (Vieira et al., 2003). We explored other markers and found that Hook was present on 31 ± 8% of wild type but only on 6 ± 2% of fob1 phagosomes, without ever decorating entire phagosomes as we observed in wild-type cells (Fig. 4 C). Interestingly, Drosophila Hook has been implicated in the maturation of multivesicular bodies (Sunio et al., 1999), which are involved in phagosome maturation (Philips et al., 2008). Considering the connection between endosomal and phagosomal maturation pathways, our data suggest that fob1 phagosomes failed to acquire late endosomal/lysosomal characteristics due a loss of fusion with those compartments.

Fob mutants exhibit a specific defect in the fusion of lysosomes with phagosomes

Several lines of evidence argue that the fob1 phagosomal maturation defect does not reflect a block in endocytic trafficking. For example, distribution of Boss and Delta ligands, which sensitively respond to loss of Vps16A or Car/Vps33A function (Fig. S2, I and J; Pulipparacharuvil et al., 2005; Akbar et al., 2009), was not altered in eye discs from fob1 larvae, indicating that endocytic trafficking proceeded normally (Fig. S2, C–H). Furthermore, eyes of 2-d-old flies exhibited normal ommatidial organization (Fig. S2, A and B), which indicates that fob1 mutants have no significant defects in Notch or EGF receptor signaling. The recurrent use of these signaling pathways during eye development provides a sensitive read-out for defects in formation of apical polarity, adherens junctions, or changes in endocytosis, lysosomal delivery, or recycling. Furthermore, starvation-induced autophagy, which requires fusion with lysosomes, is also normal in fob1 larvae (Fig. S2, L–O). Together, these data indicate that fob is not essential for endocytic or autophagic routes engaged during developmental signaling or cell remodeling and instead point to a specific requirement of fob for the fusion of phagosomes to lysosomes.

To directly test this hypothesis, we functionally labeled lysosomes by allowing hemocytes to internalize dextran by fluid phase endocytosis. After a 90-min chase in wild-type cells, 60–80% of dextran had reached lysosomes, as measured by their colocalization with LysoTracker (Fig. 4 D). This was not
Phagosomes colocalized with dextran, which indicates their failure to fuse with the prelabeled lysosomes. Because the evaluation of late-stage bacterial phagosomes is complicated by the continuous degradation of bacteria, we also tested the phagocytosis of latex beads. Here the loss of phagosome/lysosome fusion was even more evident, as after a 30–45 min chase, 50 ± 13% of latex beads colocalized with dextran in wild type, but only 8 ± 2% colocalized in \( fob^1 \) hemocytes.

Vps16B proteins in various species tightly interact with the corresponding Vps33B partners (Fig. S3 A; Pulipparacharuvil et al., 2005; Zhu et al., 2009; Cullinane et al., 2010). Consistent with a shared role of this complex in phagocytosis, dVps33B knockdown also rendered flies hypersensitive to \( E. coli \) significantly altered in \( fob^1 \) or Vps33B knockdown hemocytes, which is consistent with the notion that neither is necessary for endocytic trafficking. In contrast, in Vps16A knockdown hemocytes, dextran failed to reach lysosomes (Fig. 4 F), which suggests that lysosomal dysfunction (Pulipparacharuvil et al., 2005) rather than a defect in phagocytosis may be the primary cause of reduced bacterial clearance in Vps16A knockdown hemocytes (Fig. 2 F). Next, hemocytes containing dextran-prelabeled lysosomes were allowed to phagocytose bacteria. After a 30–45 min chase, 28 ± 7% of bacteria-containing phagosomes colocalized with dextran, which indicates their failure to fuse with the prelabeled lysosomes. Because the evaluation of late-stage bacterial phagosomes is complicated by the continuous degradation of bacteria, we also tested the phagocytosis of latex beads. Here the loss of phagosome/lysosome fusion was even more evident, as after a 30–45 min chase, 50 ± 13% of latex beads colocalized with dextran in wild type, but only 8 ± 2% colocalized in \( fob^1 \) hemocytes.

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null cells were generated in the eye discs, which were stained as described for in situ hybridization (Pulipparacharuvil et al., 2005). Instead, we found that *fob* is specifically required for fusion of phagosomes with late endosomes/lysosomes. This specificity distinguishes *fob* from other genes, such as *rab7* or genes encoding ESCRT subunits, which are required for phagosome maturation but also participate in endocytic delivery to lysosomes (Philips et al., 2008). Together, these data indicate that Vps16A and Vps16B, and their corresponding binding partners Vps33A and Vps33B, have nonredundant functions in these pathways (Suzuki et al., 2003; Lo et al., 2005; Pulipparacharuvil et al., 2005; Akbar et al., 2009).

A possible role of Vps16B and Vps33B proteins in phagocytosis is also consistent with several previous observations. Dephosphorylation of Vps33B is one among several strategies implicated in phagosome/lysosome fusion as well (Kinchen and Ravichandran, 2010). Together, these data point to the possibility that phagosome–lysosome fusion may be the ancestral function of Vps16B/Vps33B proteins, and it will be important to identify which aspects of ARC syndrome are caused by defects in phagocytosis.

**Materials and methods**

**Fly genetics and generation of *fob* mutants**

Ends-out homologous recombination was used for generating a *fob* null allele (Gong and Golic, 2003). Stepwise, left (5,906 bp) and right (5,293 bp) flanking regions of the gene were cloned into p[2w5.2] vector yielding pw5-16Bko. Primers for amplification of left and right regions were: left, 5′-ATTTGCGGCGCTGGTGGTGATGTCAC-3′ and 5′-ATTTGCGGCGCTGGTCATGTTAATGTCAC-3′; middle, 5′-GTGCTGGGACGCACTGGGACAA-3′; and 5′-GACGATGACCGCAGCTTCAC-3′. A transgenic line carrying this donor on the second chromosome was selected for targeting (Gong and Golic, 2003). Candidates for which the mini-white gene of pw5-16Bko mapping to the third chromosome were analyzed by probing Southern blots, first with the entire *fob* gene and then with *vps33B* to control for loading.

For infections and immunohistology, the following lines were used: Oregon-R, *fob*, *rpr* / *Df(3R)Tl-I*, *da-Gal4*, *uas-Myc-Fob* transgene was expressed under control of *Da-Gal4* or *Srp-Gal4* in *fob* background. Alternatively, a genomic fragment containing sequences 1.8 kb upstream and 0.8 kb of downstream of the *fob* coding region was cloned into a derivative of pCaSp4 for the generation of a transgenic line. For Vps33B-RNAi experiments, a 281-bp inverted repeat (bp 1,180–1,361 of the Vps33B mRNA; available from GenBank/EMBL/DDBJ under accession no. NM_143138.1) was cloned into a modified pFWIZ vector (Pulipparacharuvil et al., 2005) and expressed in transgenic flies under *uas-Gal4* control. Plasmids containing the *fob*, *vps33B*, and *car* cDNAs templates had been generated by the Berkeley Drosophila Genetics Resource Center.

**Infection experiments**

*E. coli* (DH5a), *amp* resistance, GFP, and *E. faecalis* cultures were grown overnight in Luria Bertani (LB) or brain heart infusion medium (BHI) medium at 37°C. Female virgin flies (5 old) were injected (Schneider et al., 2007) with 80 nl PBS containing a mean of 1,600 *E. coli* (OD600 = 0.1) or 200 *E. faecalis* (OD600 = 0.005). Sterile PBS was injected as a control. Injected flies (20 flies per vial) were reared at 25°C, 65% humidity, on yeast-molasses food. Injections were performed with a pico-injector (model PLI-188; Nikon) fitted with glass capillary needles. Injections were performed in triplicate (total of 60 flies) for each group with either of the indicated microbes and PBS control on the same day. All injection experiments were repeated 8–10 times. For each survival curve, flies were counted every 24 h, and bars represent mean values with standard deviation. Data were analyzed using the SAS software (SAS Institute, Inc.).

To determine bacterial load, flies were injected with *E. coli* (DH5a, kanamycin resistant, 5 flies per data point) and homogenized after the indicated time (Schneider et al., 2007). Serial dilutions were plated and colonies were counted for each time point. Data are plotted as boxes with whiskers. The mean is indicated with a diamond. The boxes indicate 25th and 75th percentiles; the bold line is the 50th percentile, whereas the whiskers show the complete range of the data.

For RT-PCR experiments, RNA was isolated using TRIzol (Invitrogen) according to the manufacturer’s instructions. For anti-microbial peptide measurements, RNA was isolated from five flies after injection (6 h for *E. coli* and 12 h for *E. faecalis*). *E. coli* RT-PCR was performed using a DNA-free, high-capacity cDNA reverse transcription kit (Fast SYBR Green master mix; Applied Biosystems) and a Fast Real-Time PCR system (7500; Applied Biosystems). Each data point was repeated three times beginning from injection. Values were normalized first with rp49 as an internal control and then expressed as fold change compared with flies injected with PBS as control. The following primer sets were used for amplification: *fob* left, 5′-TTAGTGGACC-GATCCTCTCG-3′; *fob* right, 5′-CACCAGTACCAATTTGTCTCTC-3′; *car* left, 5′-AACCCCTTGGCAAT-3′; and *car* right, 5′-AGTACCCACTCA-3′. qRT-PCR was performed using a DNA-free, high-capacity cDNA reverse transcription kit (Fast SYBR Green master mix; Applied Biosystems) and a Fast Real-Time PCR system (7500; Applied Biosystems). Each data point was repeated three times beginning from injection. Values were normalized first with rp49 as an internal control and then expressed as fold change compared with flies injected with PBS as control. The following primer sets were used for amplification: *fob* left, 5′-TTTAGTGGACC-GATCCTCTCG-3′; *fob* right, 5′-CACCAGTACCAATTTGTCTCTC-3′; *car* left, 5′-AACCCCTTGGCAAT-3′; and *car* right, 5′-AGTACCCACTCA-3′.

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After a 15-min incubation at 4°C, unbound bacteria were washed out and phagocytosed bacteria were chased for the various times. (a) 15 min to measure initial uptake, after which extracellular fluorescent bacteria were quenched with Trypan blue. (b) To visualize late stage phagosomes in live cells, we chased for 30 min, after which we collected images for 15 min. We call this a “30–45 min chase.” (c) To capture early or late stage phagosomes by immunofluorescence staining, we fixed the bacteria (2–4 × 10^7 ml^-1) and washed them with the indicated antibodies. (d) Temperature (21°C). All digital images were imported into Photoshop (Adobe) and analyzed using ImageJ. To measure changes in phagosome pH, fob^1 or wild-type hemocytes were incubated with double-labeled bacteria (2–4 × 10^7 ml^-1) at 4°C for 15 min. After a 10-min or 30-min chase, fluorescence ratios were measured from intact bacteria that did not appear degraded as indicated by diffuse fluorescence.

**Immunofluorescence and dextran internalization**

Hemocytes were incubated with dextran–Alexa Fluor 488 or dextran–Alexa Fluor 594 (10 kD, 1 mg/ml) for 5 min in PBS, pH 7.4, for monitoring fluid phase endocytosis. Free dextran was removed by washing extensively, and cells were chased for 90 min in Schneider’s Drosophila medium with 10% heat-inactivated FBS. After chase, cells were either incubated with LysoTracker (GFP-certified Lyso-ID red lysosomal detection medium with 10% heat-inactivated FBS) or double-labeled bacteria (2–4 × 10^7 ml^-1) at 4°C for 15 min. After a 10-min or 30-min chase, fluorescence ratios were measured from intact bacteria that did not appear degraded as indicated by diffuse fluorescence.

**matured in fob mutants and compared to the reduced lysosomal delivery after loss of Vps16A or Carnation function.**

**References**


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Wickner, W. 2010. Membrane fusion: five lipids, four SNAREs, three chap-
Supplemental material

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Figure S1. **Fluorescence ratio of Oregon green/Rhodamine double-labeled bacteria changes with pH.** A calibration curve was obtained from fluorescence ratios measured while incubating bacteria in defined pH buffers.
Endocytic trafficking and autophagy are not altered in fab1 mutants. (A and B) Semithin sections of compound eyes from 2-d-old fab1 flies (B) reveal normal arrangement of ommatidia compared with wild type (A). Ommatidial arrangement depends on normal Notch and EGF receptor signaling, which is sensitive to changes in endocytosis. (C–K) Micrographs of eye imaginal discs from larvae with the following genotypes: wild type (C–E), fab1 (F–H), DA-Gal4/Vps16A-RNAi (I), FRT19-nGFP/FRT19 car146; Hs-Flp (J), or FRT19-nGFP/FRT19 car146; Hs-Flp; tub>Rab7-YFP (K). Eye discs were stained for Boss (C, F, and I) or Delta (D, G, and J). Boss is expressed on R8 cells and endocytosed into R7 cells (arrows) during signaling. Similarly, Delta is dynamically endocytosed in many cells in the developing eye disc. Neither of these ligands exhibited any significant difference between wild-type and fab1 mutant tissues, but accumulated in vps16A knockdown (I) or car146-null cells (J). (L–O) LysoTracker staining of fat bodies was used to test induction of autophagy. No difference in autolysosomes visualized by LysoTracker was observed when comparing wild-type (L and M) and fab1 mutant (N and O) fat bodies before (L and N) or after (M and O) 4 h of protein starvation. Bars, 10 µm.
**Figure S3. Vps33B binds Fob in vivo and is necessary for normal immune defense.**

(A) Myc-Fob was expressed under Act-Gal4 control in fob1 background and immunoprecipitated using anti-Myc antibody. Expression of Myc-Fob was detected in whole fly lysates. Tubulin was used as loading control. Myc-Fob coimmunoprecipitated endogenous dVps33B, as detected in immunoprecipitated eluates with anti-Vps33B antibodies raised against a dVps33B-His6 fusion protein. UAS-Myc-Fob only (lane 1), Act-Gal4 only (lane 2), and fob1 (lane 4) without transgenes were used as experimental controls. The genotypes used were: w/w; uas-Myc-Fob/+; +/+ (lane 1), w/w; Act-Gal4/+; +/+ (lane 2), w/w; Act-Gal4/uas-Myc-Fob; fob1/fob1 (lane 3), and w/w; +/+; fob1/fob1 (lane 4). (B) Da-Gal4/Vps33B-RNAi flies exhibited reduced levels of Vps33B compared with wild type (Ore-R). Before detection on Western blots, lysates were enriched for Vps33B by immunoprecipitation with anti-Vps33B beads. (C) Survival after infection with *E. coli* was measured for flies with uas-Vps33B RNAi expressed under the control of one copy of Actin5C-Gal4, two copies of Da-Gal4, or no driver. Although two copies of Da-Gal4 were more efficient, expression with either driver caused *E. coli*-infected flies to die significantly faster when compared with control with no Gal4 driver (P < 0.001, logrank). Importantly, like fob1, flies homozygous for Da-Gal4 and uas-Vps33B-RNAi were homozygous viable and fertile, and lacked any overt developmental defects. Error bars represent mean values with standard deviation.