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Drosophila *Rab14 mediates phagocytosis in the immune response to* Staphylococcus aureus

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Abstract

Drosophila hemocytes are essential for the animal to resist *Staphylococcus aureus* infections. Phagocytosis is a central component of the hemocyte-mediated immune response. It involves regulated interaction between the phagocytic and the endocytic compartments. Rab GTPases are pivotal for the membrane trafficking and fusion events, and thus are often targets of intracellular pathogens that subvert phagocytosis. An *in vivo* screen identified Rab2 and Rab14 as candidates for proteins regulating phagosome maturation. Since Rab14 is often targeted by intracellular pathogens, an understanding of its function during phagocytosis and the overall immune response can give insight into the pathogenesis of intracellular microbes. We generated a *Drosophila Rab14* mutant and characterized the resulting immune defects in animals and specifically in hemocytes in response to an *S. aureus* infection. Hemocyte based immunofluorescence studies indicate that Rab14 is recruited to the phagosome and like Rab7, a well-characterized regulator of the phagocytic pathway, is essential for progression of phagosome maturation. *Rab14* mutant hemocytes show impaired recruitment of Rab7 and of a lysosomal marker onto *S. aureus* phagosomes. The defect in phagocytosis is associated with higher bacterial load and increased susceptibility to *S. aureus* in the animal.

Introduction

Drosophila hemocytes play an important role during the immune response against the extracellular pathogen *Staphylococcus aureus (*Nehme et al., 2011). Hemocytes are phagocytic and are functionally analogous to the mammalian macrophage. The uptake of a microbe by a phagocytic cell is followed by fusion of the microbe-containing phagosome with the early endosome, the late endosome and eventually with the lysosome, resulting in microbial clearance (Desjardins *et al.*, 1994). A comparison of the proteomes found that 70% of the mammalian phagosome proteins were also found on *Drosophila* S2 cell phagosomes (Garin *et al.*, 2001, Stuart *et al.*, 2007). Hence, much of the phagocytic machinery, that includes proteins necessary for signaling and membrane trafficking following uptake, is likely conserved between *Drosophila* and mammals.

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RabGTPases, members of the Ras superfamily of small GTPases, are required to maintain specificity during the fusion processes (Vieira et al., 2002). At different stages of maturation, phagosomes are marked by the transient association of RabGTPases, Rab5 and Rab7 (Desjardins *et al.*, 1994) which in turn are essential for progression of phagosome maturation (Alvarez-Dominguez *et al.*, 1999, Rupper *et al.*, 2001, Harrison *et al.*, 2003). A wide array of RabGTPases are associated with phagosomes (Garin *et al.*, 2001, Stuart *et al.*, 2007), and each presumably contribute to phagolysosome biogenesis. However, apart from Rab5 and Rab7, there is a limited understanding of the function of other RabGTPases in the process. Also it is not known how a defect in phagosome maturation affects the overall immune response of a host. Since hemocytes have been demonstrated to be critical against *S. aureus*, the *Drosophila* infection model provides an ideal system to gain such insights.

From a phagosome maturation screen of adults expressing dominant negative (DN) versions of each of the phagosomal RabGTPases, we identify Rab14 as a potential regulator of *S. aureus* phagosome maturation. Rab14 is known to be targeted by intracellular pathogens (Kyei *et al.*, 2006, Capmany *et al.*, 2010, Huang *et al.*, 2010, Seixas *et al.*, 2012), but its function within the cell is not well understood. Our study examines the role of Rab14 during the cellular responses in *Drosophila*. Cell biological studies indicated that Rab14 localizes to early endosomes and also, to Rab7-positive late endosomes. The localization is consistent with its likely function, as endosomal progression is impaired in the absence of Rab14. Since endocytic and phagocytic machinery overlaps, we hypothesized that Rab14 actively regulates phagosome maturation at an essential stage and this may explain why it is actively targeted by intracellular pathogens. This is consistent with our finding that Rab14 regulates fusion of phagosmes with the late endosomes and lysosomes Our study also demonstrates that Rab14 function is critical for the animal's ability to control bacterial loads during a systemic infection, thus demonstrating the central role of phagocytosis during the *Drosophila* immune response to *S. aureus*.

Results

A screen identifies Rab14 as a potential regulator of phagosome maturation

In an attempt to identify the RabGTPases that regulate phagosome maturation, we screened all of the RabGTPases associated with latex bead phagosomes that had not been previously characterized as a regulator of phagocytosis in a metazoan system (Stuart *et al.*, 2007). Drosophila has around 33 Rab genes (Zhang *et al.*, 2007) and the proteomic data indicated the presence of 12 RabGTPases on the phagosome: Rab1, 2, 4, 5, 6, 7, 8, 10, 11, 14, 18, 35. Scott and group (Zhang *et al.*, 2007) have generated transgenic fly stocks of DN- mutants of the individual RabGTPases. Utilizing those lines, we expressed DN-RabGTPases specifically in the hemocytes. Thereafter, the adult flies were assessed for the effect on phagosome maturation of pHrodo-conjugated *S. aureus.* The pHrodo dye is sensitive to pH: it is non-fluorescent at neutral pH and its fluorescence intensity increases with an increase in acidity. The progression of phagosome maturation involves fusion of a microbe-containing phagosome with increasingly acidic compartments. Thus, following uptake by the hemocytes, as maturation progresses, pHrodo-conjugated microbes show an increase in fluorescence intensity. In *Drosophila*, the hemocytes along the dorsal vessel can be

visualized through the cuticle and provide a means to monitor phagocytosis and maturation *in vivo* (Elrod-Erickson *et al.*, 2000). Rab7 is a well characterized regulator of maturation; the pHrodo conjugates of *S. aureus* displayed slower kinetics of maturation, which was notable at 60 and 90 min post-injection in adults expressing a dominant negative (DN) form of Rab7 in hemocytes (Fig. 1A).

Rab14 and Rab2 (Fig. 1B, C) were identified as potential regulators of phagocytic uptake and/or phagosome maturation in the screen. Earlier RNAi-based studies (Shim *et al.*, 2010) found no effect on phagocytic uptake of *E. coli* particles by S2 cells, after downregulation of any of the Rabs that we tested. This suggests that the demonstrated decreased pHrodo intensity in Rab2 and Rab14 DN mutants could be due to defects in maturation. Rab2 and Rab14 have been shown to regulate apoptotic cell phagosome maturation in *C. elegans* (Guo *et al.*, 2010). We chose to pursue Rab14 as little is known about is function and it has been proposed to be involved with the pathogenesis of the intracellular pathogen *M. tuberculosis* (Kyei et al., 2006).

Rab14 positively regulates maturation of phagosomes containing *Staphylococcus aureus*

The *Drosophila* genome encodes three different *Rab14* isoforms; RA, RB and RC. RNA expression analyses detected only the RA and RB isoforms in adults (Fig. S1A). The three isoforms show 81% identity to human *Rab14* and 100% identity to each other in a core region shared among all isoforms. To investigate the role of Rab14 during phagosome maturation, we generated a *Rab14* mutant. Two transposon lines, EY04032 and BG01134 conveniently flanked the *Rab14* gene, allowing the generation of a *Rab14* mutant using transposase-induced recombination (Fig. 2A). Sequencing of the interval in the mutant chromosome indicated two excision sites: one within the first transposon EY04032 and the other at the end of the second transposon BG01134. The joining of these two ends resulted in a deletion of the intervening sequence and generation of a $Rab14^{null}$ allele (Fig. 2B, C). The *Rab14* mutants showed no apparent morphological defects and were fertile.

To assess what role Rab14 might be playing in hemocytes, we examined phagocytic uptake and phagosome maturation in adult *Rab14* mutant flies. To examine phagocytic uptake, flies were injected with fluorescein-conjugated *S. aureus* and the intracellular fluorescence along the dorsal vessel was evaluated after 30 min. The uptake of fluorescein-conjugated *S. aureus* (Fig. 2D) *or E. coli* (Fig. S1B) was comparable in wildtype and the *Rab14* mutant. Quantification of the fluorescence associated with the dorsal vessel (Fig. 2E, S1C) further confirmed that Rab14 is not required for initial phagocytic uptake. This result is consistent with the work of Kim and colleagues, who carried out a phagocytosis screen to identify RabGTPases involved in the uptake of *E. coli* in *Drosophila* S2 cells (Shim *et al.*, 2010). They found that down-regulation of *Rab14* does not affect the uptake of *E. coli*.

Next, we examined phagosome maturation in *Rab14* mutant flies using pHrodo-conjugated microbial particles (Cuttell *et al.*, 2008). *Rab14* mutants showed defect at 30, 60 and 90 min post-injection of pHrodo-*S. aureus* (Fig. 2F, G). The mutant displayed slower kinetics of maturation instead of a complete block which could be due to redundancy with other RabGTPases. The defect in maturation of *S. aureus* phagosomes observed in *Rab14* mutants could be rescued with hemocyte-specific expression of Rab14 (Fig. 2H, I). Rab14 also plays

an essential role in maturation of *E. coli* phagosomes (Fig. 2J, K). Hence the phagocytic machinery seems to be conserved for these different classes of bacteria.

The rescue of the maturation defect in *Rab14* mutants by hemocyte-specific expression of Rab14 indicates its function in the hemocyte is critical for phagosome maturation. To determine if there might be differences between the Rab14 isoforms RA and RB, we examined the localization of the fluorophore-tagged proteins in larval hemocytes (Fig. S1D). Both the isoforms showed similar localization patterns suggestive of overlapping functions.

Rab14 mutant phagosomes show decreased fusion with lysosomes

The newly formed phagosome interacts with the early endosome, late endosome and then the lysosome, which leads to acidification of the phagosome (Vieira *et al.*, 2002). The recruitment of the early endosomal marker Rab5 and late endosomal marker Rab7 are essential for this sequential maturation. To understand the role of Rab14, we asked at what stage is phagosome maturation affected in *Rab14* mutant hemocytes. We examined the recruitment of Rab5, Rab7 and the lysosomal marker Spinster (Sweeney *et al.*, 2002) onto the *S. aureus* phagosome in hemocytes from wildtype and *Rab14* mutants. The recruitment of Rab5 or Rab7 is characterized by the presence of the protein on the phagosomal membrane surrounding the bacteria and can be examined by immunofluorescence. An analysis of the fluorescence intensity 3 an RGB plot of the image, wherein the endosomal marker signal is observed relative to the microbial signal, provides an unbiased means to assess co-localization. Additionally, the percent recruitment for an individual marker was calculated by taking the ratio of the number of *S. aureus* phagosomes showing recruitment of the marker over the total number of phagosomes assessed. After a 20 min pulse, phagosomes from both wildtype and *Rab14* mutant hemocytes recruited Rab5 in a comparable manner, as assessed by RGB plots of Rab5-*S. aureus* co-localization (Fig. 3A) and by the fraction of *S. aureus* phagosomes containing Rab5 (Fig. 3C). These data suggest that phagosome maturation is not affected at the early stages in the absence of Rab14. Next, we looked at Rab7-positive phagosomes after a 10 min chase following the 20 min pulse. When compared to wildtype, *Rab14* mutant cells showed significantly lower levels of Rab7 recruitment to *S. aureus* phagosomes (40.6±1.4% and 26.0±2.3% recruitment, respectively) (Fig. 3B, D). Lastly, the phagosome-lysosome fusion was assessed by examining localization of Spinster onto the phagosomes containing *S. aureus* after a 20 min chase following the 20 min pulse. Whereas relatively high numbers of bacteria showed co-localization with the lysosomal marker Spinster in wildtype hemocytes, significantly less Spinster-positive phagosomes were observed in *Rab14* mutant cells (65.0±5.4% and 35.0±7.1% recruitment, respectively) (Fig. 4A, B). These data suggest that Rab14 plays an important role in formation of phagolysosomes. The recruitment of Rab7 onto phagosomes is essential for its subsequent fusion with late endosomes and lysosomes (Rupper *et al.*, 2001). This indicates that the defect in maturation in the *Rab14* mutant could be due to the reduced fusion of phagosomes with late endosomes and lysosomes

Next, we examined the recruitment of Rab14 to *S. aureus* phagosomes in wildtype hemocytes (Fig. 4C, D). Rab14 could be found associated with *S. aureus* phagosomes just after a 20 min phagocytosis pulse or with a pulse followed by a 10 min chase. However, the

presence of Rab14 was significantly reduced on phagosomes following a 20 min chase (Fig. 4C). This timeline of Rab14 recruitment onto phagosomes is consistent with a broad role of this GTPase in regulating the maturation of phagosomes prior to the terminal fusion events that give rise to the phagolysosome.

Following uptake, similar to phagocytosis, endocytosis also involves trafficking to lysosomes, and Rab7 recruitment is essential during both endocytosis and phagocytosis (Feng *et al.*, 1995, Harrison *et al.*, 2003). Hence we asked whether Rab14 also plays a role during endosome to lysosome trafficking. We examined the expression of GFP-LAMP in the *Rab14* mutant. Following post-translational modification in the Golgi, the LAMP1-derived cytoplasmic tail is sufficient to target this fusion protein to late endosomes and then to lysosomes (Rohrer *et al.*, 1996). The GFP portion of the transmembrane fusion protein faces the lumen of the compartment. Hence the fluorescence of the fusion protein is lost as the GFP is degraded by hydrolases in the highly acidified lysosomal compartment. An impediment at any step of targeting from the Golgi to the lysosome will lead to accumulation of fluorescent vesicles in the cytoplasm (Pulipparacharuvil *et al.*, 2005). When compared to wildtype, *Rab14* mutant fat body cells accumulated significant GFP-LAMP perinuclear puncta, indicating a defect in late endosomal to lysosomal trafficking (Fig. 4E). Together with the reduced recruitment of Spinster to bacterial phagosomes in Rab14 hemocytes, these data suggest that Rab14 activity is important for regulating lysosomal trafficking in the context of both phagosomal and endosomal pathways. This raises the possibility that the primary function of Rab14 is may be in endosomal maturation. Since the phagocytic pathway interacts with the endocytic pathway, a defect in endosomal maturation could affect phagosomal maturation.

Rab14 can be localized both to early and late endosomal compartments

To determine the identity of the Rab14 endosomal compartment, we examined the co-localization of Rab14 with Rab5 and Rab7 across the whole cell. Since a Rab14 antibody is not available, Rab14-mRFP was expressed in hemocytes and immunofluorescence studies were carried out using Rab5, Rab7 and dsRed-conjugated antibody which detects mRFP. Real-time PCR studies were done to examine overall expression of Rab14 after overexpression using CgGal4 or hmlGal4; the expression data indicates Rab14 levels in these lines were comparable to wildtype (Fig. S2). Co-localization analysis on immunofluorescence images were carried out utilizing the Intensity correlation analysis software for Image J (Li *et al.*, 2004). This analysis generates a Pearson coefficient of co-localization for each cell. Pearson Co-localization Coefficient (PCC) values range from −1 to +1, corresponding to degrees of co-localization that range from exclusion (−1) to complete co-localization (+1). PCC values from 0.5 to 1 indicate the presence of co-localization (Zinchuk *et al.*, 2009). The early endosomal marker Rab5 and mRFP-tagged Rab14 showed co-localization in 56% of examined larval hemocytes with PCC reaching a maximum of +0.81 (Fig. 5A, C). A second early endosomal marker, FYVE-GFP also showed similar co-localization with Rab14, further confirming its early endosomal localization (Fig. S3). However the co-localization between Rab14 and the early endosomal markers was not ubiquitous and 44% of examined hemocytes showed low PCC values, indicating that Rab14 and Rab5 also exist in independent compartments (Fig. 5C). This is

consistent with our finding that Rab14 could also be found on Rab7-containing compartments in around 28% of hemocytes, with PCC values reaching a maximum of +0.67 (Fig. 5B, C). Hence, like its mammalian ortholog, *Drosophila* Rab14 is found on early endosomes (Junutula *et al.*, 2004). But unlike a previous report (Proikas-Cezanne *et al.*, 2006), we find that Rab14 is also found on Rab7-positive late endosomal compartments. This discrepancy could be attributed to the partial Rab14 co-localization with both markers at different stages of the maturation process in different cells. Hence, its presence on late endosomes might have been overlooked in the earlier study.

Endocytic/phagocytic machinery mutants are susceptible to *S. aureus*

To evaluate the consequences of Rab14 loss on the ability of the fly to withstand infections, we looked at the survival of *Rab14* mutants over a time period following infection with *S. aureus*. For these experiments, adult flies were injected with a log phase culture of *S. aureus* and compared to buffer-injected flies as wounding controls. *Rab14* mutant flies were significantly more susceptible to *S. aureus*, while there was no increased mortality in response to wounding (Fig. 6A, B). Also, *Rab14* mutants were not susceptible to *E. coli* infection (Fig. 6C). We also examined survival of flies upon hemocyte-specific expression of Rab7DN and observed no increase in susceptibility to *E. coli* (Fig. S4). The differential response to the two microbes could be due to the fact that *E. coli* is not pathogenic to *Drosophila.* It has been shown that a block of both the cellular and humoral responses is required to see an increase in susceptibility to *E. coli* (Elrod-Erickson *et al.*, 2000). In one instance, a complete block in maturation does result in susceptibility to *E. coli* (Akbar *et al.*, 2011), however the delayed phagosome maturation as seen in *Rab14* mutants or hemocyte-expressing Rab7DN may not be enough to completely block clearance of the microbe. Expression of either *Rab14* isoform RA or RB specifically in the hemocytes rescued the susceptibility of the *Rab14* mutant to *S. aureus* (Fig. 6B). Hence both isoforms can fulfill the immune function of Rab14 in hemocytes, suggesting that they have similar activities. Furthermore, our rescue data indicate that the increased susceptibility in *Rab14* mutants is due to Rab14 function specifically in hemocytes. To determine whether the increased death of *Rab14* mutants following *S. aureus* infection was due to defective resistance or decreased tolerance (Schneider *et al.*, 2008), bacterial loads were examined following infection by comparing colony forming units (cfu) 24h after infection. *Rab14* mutants had significantly higher *S. aureus* loads after infection and, as was the case for survival, this could be rescued with hemocyte-specific expression of either isoform (Fig. 6D). Hence the increased susceptibility of the *Rab14* mutant to *S. aureus* is due to the inability of *Rab14* deficient hemocytes to limit bacterial growth.

Hemocyte-specific expression of a Rab14DN (S49N) (Zhang *et al.*, 2007) in adults increased its susceptibility to *S. aureus* infection, confirming that the active GTPase function in hemocytes is important for the immune response against *S. aureus* (Fig. 6E). Furthermore, ubiquitous downregulation of Rab14 activity did not increase susceptibility compared to downregulation in the hemocytes alone (Fig. 6E). This corroborates that the role of Rab14 in hemocytes alone accounts for the decreased ability of *Rab14* flies to control an infection with pathogenic *S. aureus*.

Rab7 is a known regulator of phagosomal maturation. To compare the consequences of hemocyte-specific loss of Rab7 function with that of Rab14, we used hemocyte-specific expression of Rab7DN, and found that it also increased susceptibility to pathogenic *S. aureus*.(Fig. 6F). Hence our study demonstrates that the function of phagocytic machinery associated RabGTPases, including Rab14, is essential for mounting an effective immune response against pathogenic *S. aureus*.

Discussion

RabGTPases like Rab5 and Rab7 regulate phagosome fusion and perinuclear transport essential for progression of phagosome maturation (Vieira *et al.*, 2002). However the contribution of RabGTPases *in vivo* during the immune response has not been examined. We have identified Rab14 as an integral component of the phagocytic machinery and thereby essential for the *Drosophila* immune response. *Rab14* mutants show defects in *S. aureus* phagosome maturation and cell biological studies indicate that Rab14 is required for efficient recruitment of Rab7 on to the bacterial phagosome and is itself recruited to phagosome on early and late phagosomal compartments. A similar temporal profile for Rab14 recruitment, following Rab5 and preceding Rab7 has been shown in *C. elegans* during apoptotic cell phagosome maturation (Guo *et al.*, 2010). This finding points to a role for Rab14 during the Rab5 to Rab7 transition. In contrast to our findings, a prior study in murine macrophages indicated that Rab14 is a negative regulator of phagosome maturation, as overexpression of wildtype Rab14 or a GTPase-deficient, constitutively active (CA) Rab14 inhibited maturation of phagosomes containing dead *Mycobacteria* (Kyei *et al.*, 2006). The contradiction may be due to the nature of those experiments. The appropriate regulation of the active-inactive switch of RabGTPases is critical for its function, so a constitutively active (CA) mutant might result in a gain-of-function phenotype. For instance, although Rab5 regulates early endosome fusion and is essential for the endocytic pathway (Bucci *et al.*, 1992), overexpression of wildtype or CA Rab5, leads to continuous fusion between early endosomes, resulting in large endosomes with early endosome characteristics (Roberts *et al.*, 1999). Also, continuous association of active Rab5 with phagosomes results in maturation arrest (Vieira *et al.*, 2003). Thus a loss-of-function mutant provides a more accurate assessment of the overall role of a Rab GTPase.

A timeline proteomic study of latex bead phagosomes indicated biphasic enrichment of Rab14 unlike the single phase enrichments of Rab5 and Rab7 onto the phagosomes (Rogers *et al.*, 2007). Another proteomic study utilizing murine macrophages indicates the presence of a common Rab7 and Rab14-positive compartment (Duclos *et al.*, 2011). Our data fits the biphasic model of enrichment as we could localize Rab14 on both Rab5 and Rab7 positive endosomes. The functional implication could be that Rab14 is working with both Rab5 and Rab7 to maximize the efficiency of endosomal and phagosomal maturation. This is also consistent with the observation that in the absence of Rab14, maturation is not completely abolished, but instead displays slower kinetics. Also, the viability of $Rab14^{null}$ animals is not compromised, while a more severe defect in endocytic machinery would be expected to be lethal.

Due to their role in membrane trafficking, RabGTPases are often targeted by intracellular pathogens which actively arrest maturation to avoid the lysosomal killing process (Brumell *et al.*, 2007). For instance, Rab5 (Kelley *et al.*, 2003) and Rab14 (Kyei *et al.*, 2006) are actively retained for an extended period of time on mycobacterial phagosomes which do not mature into Rab7-positive compartments (Via *et al.*, 1997). Since Rab7 is excluded from the *Mycobacterium* phagosome, the extended recruitment of Rab14 could lead to homotypic early phagosome fusion leading to a delay in the formation of the late phagosome, thus aiding maturation arrest. A Rab14-related RabGTPase, RabD regulates phagosome-phagosome fusion in *Dictyostelium,* (Harris *et al.*, 2002). Homotypic phagosome fusion is unique to *Dictyostelium* phagosome maturation (Duhon *et al.*, 2002). However in mammalian cells, intracellular pathogens promote homotypic phagosome fusion as a survival strategy (Brumell *et al.*, 2007). Our data indicate that Rab14 is recruited to the early phagosomes and regulates Rab7 recruitment during phagosome maturation. It is possible that *Mycobacteria* interact with Rab14 to limit Rab7 recruitment, and in the absence of Rab7, Rab14 promotes early phagosome fusion leading to maturation arrest. Our study demonstrates the functional importance of Rab14 during phagosome maturation and the immune response and this may explain why it is specifically targeted by some pathogens.

Lastly, there is a limited understanding of the process and function of phagosome maturation in *Drosophila*. The removal (Charroux *et al.*, 2009, Defaye *et al.*, 2009) or the functional disabling of hemocytes (Nehme *et al.*, 2011) both increased fly susceptibility to *S. aureus,* but the underlying mechanism behind the observation was not understood. In this study, we demonstrate that the hemocyte exerts its influence by regulating phagosome maturation which is essential for bacterial clearance.

Materials and Methods

Fly genetics

The *Rab14* mutant was generated by transposase-induced recombination in males (Chen *et al.*, 1998, Parks *et al.*, 2004). Genomic DNA from the Rab14 locus was amplified by PCR using the following primers:

1. Forward (For): 5′-ACTGTGCGTTAGGTCCTGTTCA-3′
2. Reverse (Rev): 5′-ATGCCACCGAAGATGCTAGCTCAG-3′

The sequencing of the fragments indicated a deletion between +207bp and +3402bp within the 3617bp long *Rab14* gene.

For the rescue experiments, Rab14 RB was amplified from cDNA and a fusion with mRFP at the N-terminus was cloned into pUASt. Transgenic flies were generated in a *w1118* background (BestGene Inc). Plasmids containing mRFP were a gift from Henry Chang (Purdue University).

Flies used in the experiments: $Rab14^{EY04032}$, $Rab14^{BG01134}$, Rab14RA-YFP, RabGTPaseDN, hmlΔGAL4, cgGAL4, actinGAL4 were obtained from Bloomington. Other lines Rab5-GFP, (M. Gonzáles-Gaitán), tubulin>GFP-LAMP (H. Kramer), Spinster-GFP (G. Davis) were also generous contributions.

Bacteria strains

Staphylococcus aureus – ATCC12600

Adult phagocytosis and phagosome maturation experiments

Phagocytosis—5–7 day old adult flies were injected with fluorescein-conjugated *S. aureus* resuspended in PBS (Invitrogen: S2851, 1.6mg/ml) using a Pneumatic PicoPump PV820 (World Precision Instruments). After 30 min, flies were again injected with Trypan Blue to quench extracellular fluorescence. Images of the dorsal vessel were obtained using a Zeiss stereomicroscope (Discovery V8) with AxioCam Hc camera. Quantification of fluorescence intensity in an area around the dorsal vessel was carried out using Axiovision 4.7. Background fluorescence was also quantified and the ratio was used to plot the graph. The unpaired t-test was used to calculate p-values.

Phagosome maturation—5–7 day old adult flies were injected with pHrodo-conjugated *S. aureus* resuspended in PBS (Invitrogen: A10010, 8mg/ml). pHrodo conjugates were stored in small aliquots at −20°C. Single aliquots were used for one line to minimize light associated sensitivity. Images were taken at different time points after injection as described above.

Phagosomal marker recruitment and co-localization experiments

Phagosomal marker recruitment—Around 8–10 wildtype and *Rab14* mutants feeding 3rd instar larvae were injected with Alexa-fluor (AF) conjugated *S. aureus* (Invitrogen: S23371, S23372). The pulse of 20 min included 13 min *in vivo* and 7 min *ex vivo* phagocytosis. It included 3 min for the bleeding of hemocytes onto poly-lysine coverslips containing Schneider's media (SM) followed by 4 min of *ex vivo* incubation. Following the pulse, media was replaced with fresh media and hemocytes were incubated for the required chase time. Afterwards hemocytes were fixed in 4% paraformaldehyde and mounted in Prolong containing DAPI (Invitrogen: P36935). The hemocyte drivers hmlΔGAL4 (Goto *et al.*, 2001) or cgGAL4 (Asha *et al.*, 2003) were used to drive expression of tagged phagosomal markers. For Rab7, rabbit anti-Rab7 (1: 3000, a gift from P. Dolph, Dartmouth College, Chinchore et al., 2009) and a secondary goat anti-rabbit antibody labeled with AF-488 were used.

Co-localization experiments—Hemocytes expressing Rab14RB-mRFP (CgGAL4) were bled out from feeding third instar larvae followed by fixing and antibody staining. The following primary antibodies were used: rabbit anti-Rab5 (1:500, ab31261), rabbit anti-Rab7, goat anti-DsRed, (also detects mRFP, 1:500, sc-33354). The following secondary antibodies were used: donkey anti-rabbit IgG-CFL 488 (1:200, sc-362261), mouse anti-goat IgG-TR (1:200, sc-3916).

Endosomal maturation

Fat body was dissected out from wildtype and *Rab14* mutant larvae expressing tubulin>GFP-LAMP. The dissections were carried out in cold PBS and fat body was fixed

for 30 min in 4% paraformaldehyde. Thereafter the tissue was washed three times for 10 min and mounted in Prolong containing DAPI.

Image acquisition

Images were acquired using a Zeiss LSM 710 (100x/1.4 Oil Plan Apochomat lens or 40x/1.3 Oil Plan NeoFluar). Acquisition software Zen 2000 / LSM for Zeiss 710 was used. For publication, pictures were imported into Adobe Photoshop and adjusted for gain and contrast settings. Images kept in the same panel for comparison were treated alike.

Reverse Transcription and Quantitative Real-Time PCR

RNA was isolated by homogenizing tissue in STAT-60 buffer according to the manufacturer's protocol (Isotex Diagnostics). The RNA was digested with RNase-free DNase, then subjected to reverse transcription (Fermentas, K1622) and quantitative real-time PCR using LUX probes (Invitrogen) or SYBR green on an ABI 7300 following the manufacturers' protocols (Fermentas: K0232, K0252). Paired t-test was used to calculate p-values for differences in induction.

Primer Sequence

SYBR primers

Rab14RA: For :5′CAAACAACAATACGCACACATAC3′,

Rev: 5′GAATGTGTAACGTAGGGCGGTTA3′

Rab14 RB: For: 5′ GGACATTCAAATGAGGAGCTGAT3′,

Rev: 5′TCATCTTGACACCGGCAGAA3′

Rab14 RC: For: 5′CTATATACTCAATGACTCTGCAATGTAATA,

Rev: 5′TATGGCGCTGCAGTCATGT

Control RP49: For: 5′GCAAGCCCAAGGGTATCGA3′,

Rev: 5′TAACCGATGTTGGGCATCAG3′

Survival and bacterial load experiments

Survival—Adult flies, 5–7 day old were injected with logarithmic phase culture of *S. aureus* or *E. coli* (final OD 0.5). Flies injected with PBS served as a wounding control. The survival after injection was assessed every 24 h. At least thirty flies were injected for each experiment, and the experiments were repeated at least 3 times. Log rank tests were used to test for significant differences in survival curves, and p-values of < 0.05 were deemed significant.

Bacterial load—Adult flies, 5–7 day old were injected with a log phase culture of *S. aureus* (final OD 0.2) resuspended in PBS. For sample collection, flies were surface sterilized with 70% ethanol, washed in PBS and homogenized with a pestle in Luria-bertani media containing 1% Triton X-100. Five sets of 2–3 flies were collected and pooled for each time point. cfu/fly was calculated by serial dilution and plating on Luria-bertani agar plates.

The experiment was repeated at least 3 times. The Mann-Whitney test was used to calculate p-values.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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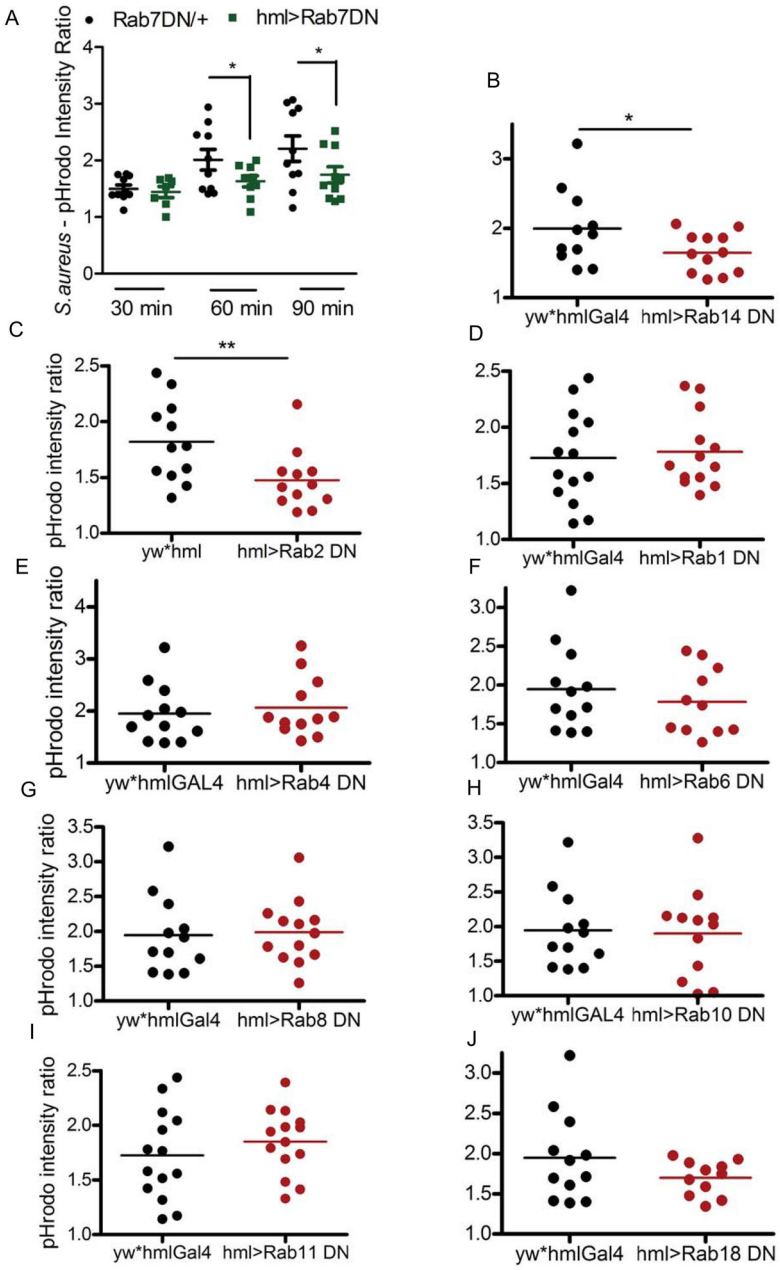

Figure 1. Identification of regulators of phagosome maturation

(A) Rab7 was used to validate the pHrodo-based phagosome maturation assay. The Rab7DN line was outcrossed to either w1118 (control) or hmlGAL4 for hemocyte-specific expression of the mutant protein. Adult flies were injected with pHrodo-*S. aureus* and examined at 30, 60 or 90 min post-injection. The fluorescence intensity in an area around the dorsal vessel and background fluorescence was quantified, and the ratio was used to plot the scatterplot. Each dot represents the intensity associated with one fly. For the screen, adult flies were injected with pHrodo *S. aureus* and imaged after 60 min. The DN lines were crossed to hmlGAL4. The parental line of the dominant-negative expressing line (yw) outcrossed to the

hemolectin driver line was used as the control. Representative dot plots for: (B) Rab14, n=6 (C) Rab2, n=4 (D) Rab1, n=3 (E) Rab4, n=3 (F) Rab6, n=3 (G) Rab8, n=5 (H) Rab10, n=3 (I) Rab11, n=3 and (J) Rab18, n=3 are shown. n= number of experimental replicates * $p < 0.05$ ** $p < 0.01$.

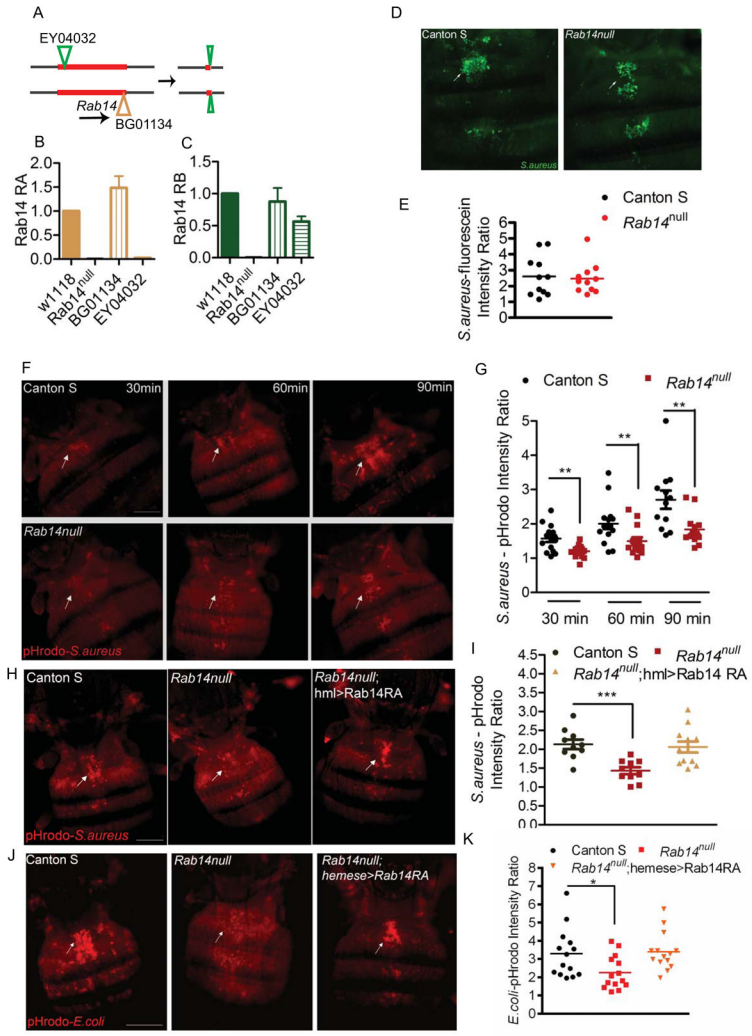

Figure 2. Rab14 is required for phagosome maturation of *S. aureus*

(A) Transposase-induced recombination between the transposons EY04032 and BG01134 in males led to the generation of a *Rab14* mutant. Flies carrying the two Rab14 transposon insertions, EY04032 and BG01134, and a transposase-expressing transgene were generated. Transposase-induced mitotic recombination in males (Chen *et al.*, 1998) led to the generation of a chromosome lacking Rab14. After mating, male flies were screened to select for recombined chromosomes using phenotypic markers associated with the two transposons. The potential null mutants were screened and a *Rab14* mutant was identified. The genomic DNA between the two transposons was lost from the mutant along with the transposon BG01134. However a small segment of EY04032 was retained in the mutant. (B, C) The Rab14 expression is not detectable in the *Rab14* mutant indicating it is a null allele. Quantitative-real time PCR was used to examine expression of Rab14 isoforms (B) RA and (C) RB in wildtype, $Rab14^{null}$, BG01134 and EY04032 mutant adults. (D, E) *Rab14* mutants are not affected for phagocytic uptake of *S. aureus*. Representative images and quantification of fluorescence intensity of wildtype and the *Rab14* mutant to examine the uptake of fluorescein-conjugated *S. aureus* 30 min post injection. (F, G) Rab14 is a positive

regulator of phagosome maturation. The phagosome maturation assay was carried out in wildtype and *Rab14* mutants as described for Rab7DN mutants. (F) A representative image for each timepoint and (G) a scatterplot of the quantified images is shown. Rab14 function in the hemocyte is essential for phagosome maturation. Rescue of (H, I) *S. aureus* or (J, K) *E. coli* phagosome maturation in the *Rab14* mutant was examined upon hemocyte-specific expression of Rab14 ($Rab14^{null}$; hml>Rab14RA or $Rab14^{null}$; hemese>Rab14RA). For rescue, the flies were imaged 60 min postinjection. Phagocytosis experiments were repeated at least 3 times with 8–12 flies in each experiment. The arrows indicate dorsal vessel-associated hemocytes which were examined and quantified. Data was analyzed by unpaired t-test and asterisks on the graphs indicate p-values of significance: * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Scale bar, 0.2mm

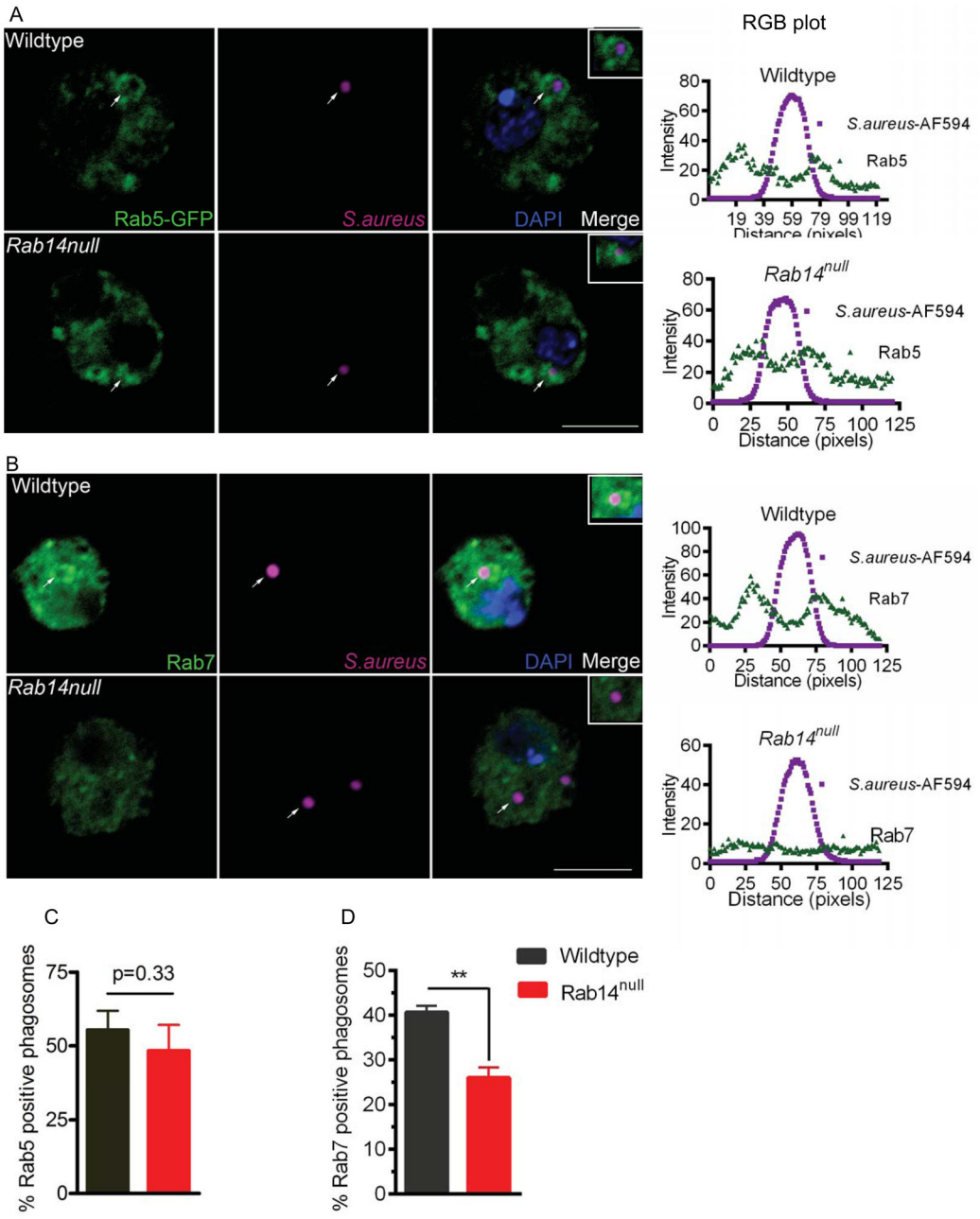

Figure 3. *Rab14* mutant phagosomes demonstrate reduced recruitment of a late phagosomal marker

(A) Wildtype and *Rab14* mutant phagosomes show comparable recruitment of the early endosomal marker Rab5. Hemocytes expressing Rab5-GFP (hmlGAL4) were bled out and fixed from wildtype and *Rab14* mutant larvae after a 20 min pulse with *S. aureus* conjugated to AF-594 (magenta). Hemocytes were evaluated for Rab5 (green) recruitment onto phagosomes. The merged image includes Rab5 (green), *S. aureus* (magenta) and DNA/DAPI (blue). Enrichment of marker-associated fluorescence in the vicinity of bacteria was considered positive for recruitment. An RGB plot for a 120 pixel line across the phagosome was plotted using image analysis software (ImageJ). The arrows on the merged image

indicate the phagosome of interest utilized to make the RGB plot. (B) *Rab14* mutant phagosomes show a reduced presence of Rab7. A 20 min pulse with *S. aureus*-AF-594 followed by a 10 chase with Schneider's media was given to wildtype and *Rab14* mutant hemocytes which were then immunostained for Rab7. Rab7 recruitment onto the phagosomes was analyzed. (C, D) Percentages of phagosomes showing recruitment of (C) Rab5 and (D) Rab7 are plotted for wildtype and $Rab14^{null}$. The experiments were conducted 3 times with 10–12 larvae in each experiment. Images were obtained using confocal microscopy and a total of 70–90 phagosomes were examined for quantification. Error bars indicate standard error of the mean (SEM), n=3. Data was analyzed by two tailed, paired t-test. ** $p<0.01$. Scale bar, 5μm

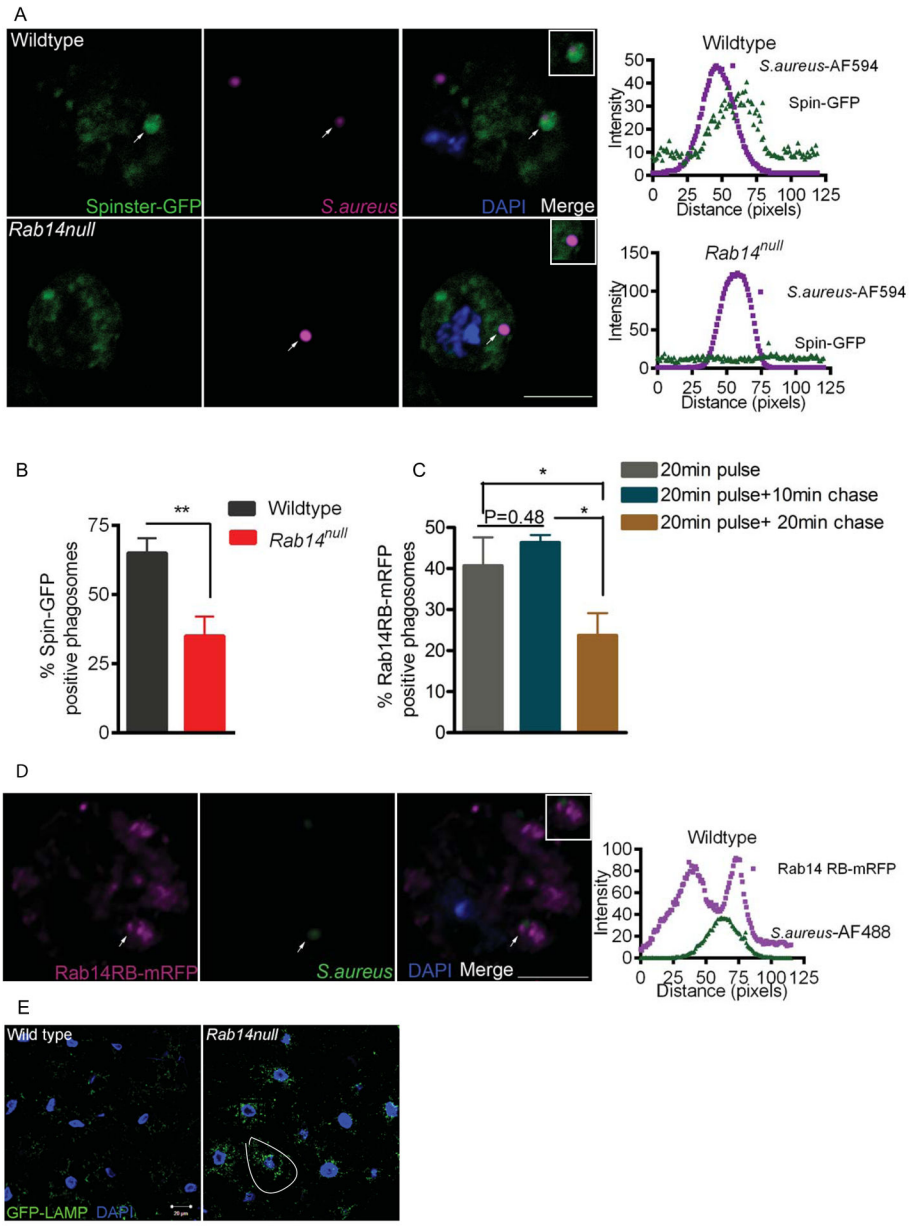

Figure 4. Rab14 is recruited to the phagosome and is required for efficient phagolysosome biogenesis

(A) *Rab14* mutant phagosomes show reduced fusion with lysosomes. Hemocytes expressing the lysosomal marker Spin-GFP (hmlGAL4) were bled out and fixed from wildtype and *Rab14* mutant larvae after a 20 min pulse with *S. aureus* conjugated to AF-594 (magenta) followed by a 20 min chase. Hemocytes were evaluated for co-localization between Spinster-GFP and phagosomes as described previously. (B) Percentage of phagosomes showing recruitment of Spin-GFP is plotted for wildtype and *Rab14*^{null} (n=4). (C) Rab14 is recruited to the phagosome. Transgenic UAS-Rab14-mRFP flies were generated and the transgene was expressed in hemocytes using cgGal4. Recruitment of Rab14-mRFP (magenta) after different chase times following an initial 20 min pulse with *S. aureus*-AF488 (green) was examined in wildtype hemocytes (n=3). (D) A representative image and RGB

plot for the 20 min pulse + 10min chase time point is shown. The experiments were conducted at least 3 times with 10–12 larvae in each experiment. A total of 70–90 phagosomes were examined for quantification. Error bars indicate SEM. Data was analyzed by two tailed, paired t-test. ** $p<0.01$ * $p<0.05$. Scale bar, 5 μm. (E) *Rab14* mutants show impaired late endosome to lysosome trafficking. Fat body from wildtype and *Rab14* mutant larvae expressing GFP-LAMP (tubulin GAL4) were dissected out and fixed. *Rab14* mutant fat body cells accumulate perinuclear GFP-LAMP puncta which indicates a defect in late endosome to lysosome trafficking. A single cell showing increased perinuclear puncta has been circled. The experiments were repeated at least 3 times with 6–7 larvae for each experiment. Scale bar, 20 μm.

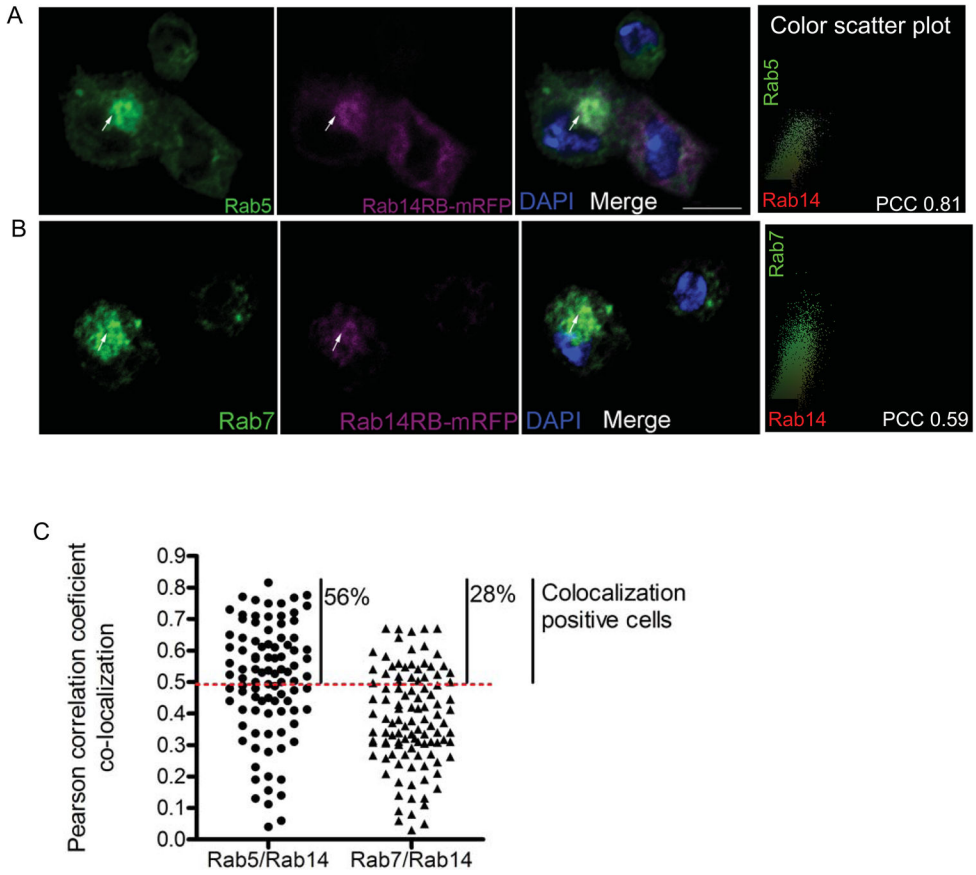

Figure 5. Rab14 co-localizes with early and late endosomal markers

(A, B) Hemocytes expressing Rab14-mRFP (cgGAL4) were bled out, fixed and immunostained for either (A) Rab5 or (B) Rab7. The arrows indicate the intracellular compartments showing co-localization. The color scatterplot for the marked cell was generated using Intensity Correlation analysis (Image J). (C) Pearson correlation coefficients (PCC) for co-localization between Rab5 and Rab14, or Rab7 and Rab14 were obtained using Intensity Correlation analysis (Image J) for each cell and plotted. PCC values from 0.5–1 indicate positive co-localization and the percentage of cells showing co-localization is indicated in the graph. A total of 90–100 hemocytes were examined for co-localization.

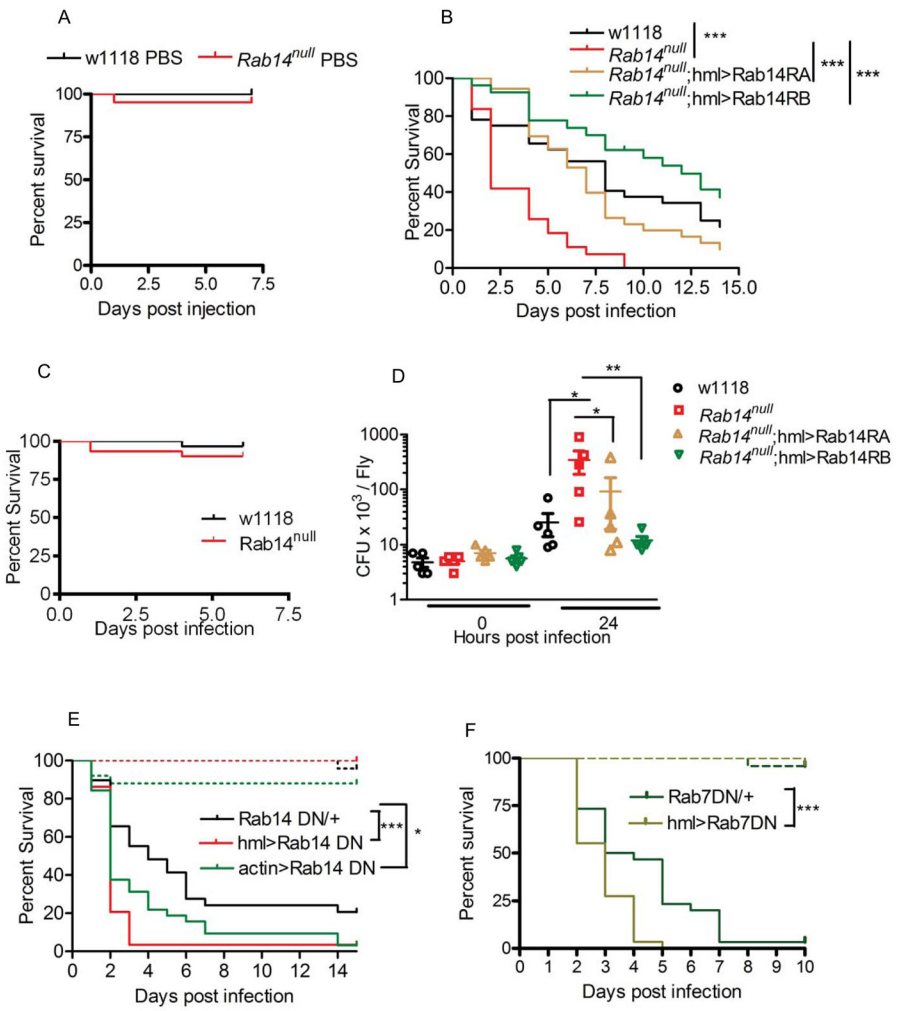

Figure 6. Phagocytic machinery mutants show increased susceptibility to *S. aureus.*

(A) *Rab14* mutants show increased mortality following *S. aureus* infection. Wildtype and *Rab14* mutant adults were injected with (A) sterile PBS (B) a log phase culture of *S. aureus* or (C) *E. coli* and survival was examined over a period of time. (D) *Rab14* mutants have high bacterial loads following infection. Adults were injected with a log phase culture of *S. aureus* and bacterial counts were evaluated by plating homogenized adults and counting colony forming units (cfu) immediately or 24h post infection. (B, D) The increased susceptibility of *Rab14* mutants to *S. aureus* is due to a hemocyte-specific role of Rab14. (B) Survival and (D) bacterial count rescues were carried out by hemocyte-specific expression (hml) of *Rab14* RA or RB in *Rab14* mutants. (E) A functionally active Rab14 in hemocytes is essential to resist *S. aureus* infection. Adults expressing Rab14DN either ubiquitously (actinGAL4) or in hemocytes (hmlGAL4) were injected with a log phase culture of *S. aureus* for survival studies. The Rab14DN line outcrossed to w1118 was used as a control. (F) Rab7 is essential for the immune response to *S. aureus*. Adults with hemocyte-specific expression of Rab7DN were injected with *S. aureus* and survival over a time period was examined. The Rab7DN line outcrossed to w1118 was used as a control. The dashed and solid lines in the survival curve represent wounding control (PBS injection) and infection

respectively. Each survival study was repeated at least 3 times with a minimum of 30 adults /line for individual experiments. The survival data and bacterial count data were analyzed by log rank tests and Mann Whitney tests respectively. * $p<0.5$, ** $p<0.01$, *** $p<0.001$.