ABSTRACT

Title of Document: ROLE OF RAB14 DURING PHAGOSOMAL MATURATION AND ITS IMPLICATION IN THE *DROSOPHILA MELANOGASTER* IMMUNE RESPONSE

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Phagocytosis is a vital component of *Drosophila* cellular immunity. The sequential interaction of a phagosome with the endocytic compartments entails docking and fusion and requires the role of many RabGTPases. We identified Rab14 as a possible regulator of phagosome maturation in an *in vivo* screen. Expression studies indicated the presence of Rab14 in hemocytes in both larval and adult stages. To further characterize the role of Rab14 during phagocytosis, a $Rab14^{null}$ mutant was generated. The *Rab14* mutant displayed no difference in the phagocytic uptake of *E.coli* or *S.aureus*, however the kinetics of phagosome maturation for both microbes was decreased. Cell biology studies indicated delayed acquisition of the late endosomal marker, Rab7 and the lysosomal marker, Spinster, on the *S.aureus* phagosome. Since recruitment of the early endosomal marker Rab5

was not affected, we concluded Rab14 functions downstream to Rab5 and is essential for efficient Rab7 recruitment on microbial phagosomes. Rab14 itself was recruited during both the early and late phagosomal stages and in accordance with that, it showed colocalization with both Rab5 and Rab7 on endosomes. *Rab14* mutants also showed a defect in endosomal maturation, suggesting Rab14 is also essential during endocytosis. *Rab14* mutants demonstrated defects in the induction of the specific antimicrobial peptides Cecropin and Defensin in response to *S.aureus* infection, indicating a possible crosstalk between the cellular and humoral response. Along with that, *Rab14* mutants also showed defects in the expression of the Toll ligand Spatzle, in response to infection. Hence we propose Rab14 regulates expression of specific antimicrobial peptides through the cytokine Spatzle. The inefficient cellular and humoral responses result in increased susceptibility of *Rab14* mutants to bacterial infection. This could be rescued by hemocyte-specific expression of *Rab14*, suggesting its role in the hemocytes is essential for the immune response against *S. aureus.* Our study identifying a role for Rab14 during phagosomal maturation gives insight on the pathogenesis of *M.tuberculosis*, a pathogen which manipulates Rab14 to subvert phagosome maturation. Moreover it identifies Rab14 function during cellular and humoral responses as an integral component of the *Drosophila* immune response against *S.aureus*.

ROLE OF RAB14 DURING PHAGOSOMAL MATURATION AND ITS IMPLICATION IN THE *DROSOPHILA MELANOGASTER* IMMUNE RESPONSE.

By

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Dedication

To my Parents

Rajiv and Neelima

my husband Amit

and daughter Aanya

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Table of Contents

List of Tables

List of Figures

Chapter 1

Introduction

I. Drosophila innate immunity

Living organisms protect themselves against a wide range of pathogens via specialized systems dedicated to surveillance against microbes. There are two main classes of immune response: the innate immune response and the adaptive immune response. The former serves as the initial line of defense and it identifies pathogen associated molecular patterns (PAMPs): Peptidoglycan, flagella, CpG DNA are some examples of PAMPs. Thus innate immunity is not specific to a particular microbe. The latter recognizes specific microbe associated antigens and hence displays high specificity. Another defining feature of the adaptive immunity is the immunological memory, by which a repeated exposure leads to a rapid clearance of the pathogen on later encounters. Innate immunity is a defense feature of virtually all the living organisms, while adaptive immunity is restricted to the jawed vertebrate group.

Drosophila melanogaster, an invertebrate has only innate immunity. The innate immune response is comprised of humoral and cellular responses. In the beetle, almost 99% of an initially high dose of *S.aureus* was cleared within the first half hour by the cellular response (Haine et al., 2008). A central component of the cellular response is phagocytosis. The humoral response is

the other arm of defense and it leads to secretion of antimicrobial peptides (AMPs) in the hemolymph. The humoral response peaks 24 h after infection, hence only microbes which escaped the action of the blood cells are cleared by the AMPs. This suggests in invertebrates, the cellular and humoral responses act synergistically to defend from microbial invaders and that the cellular response is likely the first and quite effective responder. The myriad of genetic tools available in *Drosophila* and the ease with which *in vivo* studies can be done should elucidate the role of genes regulating the cellular and humoral responses.

II. Cellular Response

The cellular response is relatively less explored. The cellular response is initiated by the hemocytes or the blood cells of Drosophila. They participate in phagocytosis, encapsulation and melanization. The body cavity of *Drosophila*, like that of other arthropods, is filled with hemolymph which has free floating and sessile hemocytes. The hemocytes can be divided into three categories: Plasmatocytes (90-95%), crystal cells (5%) and lamellocytes. The plasmatocytes get rid of dead cells and microbes by phagocytosis. Crystal cells release oxidoreductase and proPhenoloxidase (proPO), which mediate melanization at the wound site. The lamellocytes are larger adherent cells and function in the encapsulation of large objects. They are not found in embryos or healthy larvae but they can be induced upon pupariation and also upon infection in larvae with a parasitoid wasp egg.

A. Phagocytosis

Phagocytosis is the process by which blood cells recognize and actively engulf the microbe and thus is the immediate line of defense once the microbe breaches the host epithelial barrier. The same machinery also serves to clear apoptotic cells generated during development. Phagocytosis is initiated once a pattern recognition receptor (PRR) on the hemocyte recognizes the pathogen associated molecular patterns (PAMPs) of a microbe. Soluble PRRs serve as opsonins that bind to the microbe and increase the efficiency of phagocytosis.

B. Sensing the Microbe: The Receptors

The blood cell arsenal includes a great variety of receptors showing different levels of specificity. The receptors present on the plasma membrane of hemocytes bind to the microbe, leading to formation of pseudopods, followed by engulfment of the microbe. Following is a brief description of the receptors identified in *Drosophila*.

Scavenger receptors (SR): Scavenger receptors bind acetylated and/ or oxidized low density lipoprotein (LDL). Several mammalian scavenger receptors have been identified for their role in phagocytosis of apoptotic cells and microbes (Gough and Gordon, 2000). Due to dearth of known membrane bound PRRs in *Drosophila*, Ezekowitz and group (Ramet et al., 2001) initiated a study to identify phagocytic receptors in *Drosophila*. They used *Drosophila*

S2 cell lines as their model after establishing that its phagocytic potential was comparable to *Drosophila* hemocytes. In cross competition phagocytosis experiments, fluorescent labeled *E.coli* could be competed out with unlabeled *S.aureus* and vice versa. The same was not true for *Candida silvatica*, suggesting the presence of some common receptors for G+ and G- bacteria that differ from fungal receptors. Interestingly acetylated LDL which is a ligand for SRs also reduced phagocytosis of both *E.coli* and *S.aureus* by competing with the binding of the microbe to the cell. The dose dependent inhibition of phagocytosis by LDL suggested SRs might function as receptors on S2 cells. Still, the LDL did not completely abrogate the phagocytosis, pointing to the likely presence of more than one type of receptor for each microbe. Taking a cue from the role of mammalian class A SRs in phagocytosis, the group examined the role of the dSRs in S2 cell phagocytosis. The tissue specificity and the temporal expression profile of dSRs CI, CII, CIII and CIV indicated dSR-CI as a potential candidate, since the hemocyte-specific expression through all the development stages coincided with the functional requirements of a receptor. An in-vitro binding assay with dSR-CI expressed in COS cells confirmed its role in binding of *E.coli* and *S.aureus*. Furthermore, in a FACS analysis, RNAi-mediated gene silencing of dSR-CI in S2 cells decreased the cell associated (bound and internalized) *E.coli* and *S.aureus* by 20 and 30% respectively. This incomplete obliteration of phagocytosis further supported the hypothesis that more than one receptor is involved in the uptake of a microbe.

Mycobacterium infections in *Drosophila* nicely model mycobacterial infection in humans (Dionne et al., 2003). In an S2 cell screen done to identify host components regulating *M. fortuitum* infection, a class B scavenger receptor *peste* was identified (Philips et al., 2005). An RNAi screen was carried out to select for genes that could lower the level of *M. fortuitum* infection in S2 cells as assessed by expression of two mycobacterial promotor (*map)*-reporter (GFP) fusions. Down-regulation of the receptor *peste* decreased the infection by *M.fortuitum* by 60%, suggesting its uptake is regulated by *peste*. The heterologous expression of *peste* in normally *M.fortuitum*-refractory human embryonic kidney 293 (HEK293) cells led to uptake of mycobacteria into the cells, further confirming its role as a receptor. While down-regulated *peste* did not affect *E.coli* and *S.aureus* uptake in S2 cells, the heterologous expression in HEK293 did support uptake of *E.coli* and *S.aureus*. It is possible that the presence of redundant receptors for *E.coli* and *S.aureus* in S2 cells masks a phenotype for *peste*. Another RNAi screen by the same group identified *peste* as a receptor for *Listeria monocytogenes* too (Agaisse et al., 2005). It is interesting that the same receptor has been identified for two different intracellular pathogens. Since downregulation of *peste* in S2 cells leads to a detectable difference in the uptake of the intracellular pathogens *Mycobacteria* and *Listeria*, but not of *E.coli* and *S.aureus*, it is possible there are fewer receptors for intracellular bacteria. Since these bacteria are known to be intracellular, it could be a host

mechanism to limit hemocyte infection. Alternatively, intracellular microbes might have selected *peste* because of some intresting properties.

Croquemort, a CD36 family Class B Scavenger Receptor was initially identified as a receptor for apoptotic cells in Drosophila (Franc et al., 1999). Moore and group (Stuart et al., 2005) did an extensive analysis of the role of Croquemort in phagocytosis using S2 cells and CD36 null mice. RNAi of *croquemort* in S2 cells decreased the phagocytosis of *S.aureus* by 35%. A *croquemort* null obtained through two overlapping deletions is lethal in flies, so the authors were unable to characterize the mutant. They continued the study with the mammalian paralog CD36. Expression of CD36 in HEK293T cells conferred both binding and internalization of *E.coli* and *S.aureus*, although the effect was more pronounced for *S.aureus*. Macrophages from CD36 null mice showed reduced phagocytosis for *S.aureus* (the decrease in phagocytosis was comparable to the decrease in S2 cells) and reduced cytokine production in response to *S.aureus* and lipoteichoic acid (LTA). The effect on cytokine production suggested that CD36 is cooperating with Toll-like receptors (TLR) which are also membrane-bound and regulate cytokine production. The authors further dissected the CD36 protein structure and found that its carboxy terminal domain is required for TLR2/6 signaling in response to LTA.

Thus the Scavenger receptor family in Drosophila serves as Pattern recognition receptors for extracellular microbes *E.coli*, *S.aureus* as well as intracellular pathogens like *Mycobacteria* and *Listeria*. Also *Drosophila*

studies were able to give insight to an mammalian immune response (eg., CD36).

Nimrod superfamily of receptors: This superfamily has been recently characterized and is categorized by having a variable number of NIM repeats (Kurucz et al., 2007). A NIM repeat is similar to an EGF repeat, but is shifted one cysteine unit from a normal EGF repeat consensus, so it is also known as an EGF-like repeat. The EGF-like repeat is a protein domain that plays a role in adhesion and receptor-ligand interactions. There are two broad categories in the NIM superfamily: proteins containing many NIM repeats like Eater and Nim-CI, and proteins encoded by Draper-type genes like Draper and Nim A. The latter group is characterized by one NIM domain, a variable number of EGF repeats and an EMI domain which is a possible protein-protein interaction module. Unlike the Eater group which is only restricted to insects, the Draper group is also found in *C.elegans* and human.

The observed redundancy in S2 cells for *E.coli* and *S.aureus* phagocytosis has led researchers to identify new receptors. Serpent regulates the binding and phagocytosis of bacteria in S2 cells, thus its target genes may encode genes important for the process (Ramet et al., 2002). A microarray analysis of cells downregulated for a GATA transcription factor, Serpent was carried out by Ezekowitz and group (Kocks et al., 2005).

One of the genes downregulated in the microarray study was NIM-repeat containing single pass transmembrane (TM) protein which the authors

named *eater*. Its knockdown resulted in significant down-regulation in binding and phagocytosis of *E.coli* and *S.aureus* in S2 cells. The in situ hybridization studies in wild type and $hop^{Tum\text{-}l}$ (a JAK kinase gain of function mutant leading to overproliferation of hemocytes) (Hanratty and Ryerse, 1981) flies indicated that eater is expressed only in the hematopoietic tissue. Additionally, its expression is restricted to phagocytic plasmatocytes and is excluded from lamellocytes and crystal cells. In vitro translation of the *eater* RNA showed a high level of diversity in terms of amino acid variation and EGF-like repeat length in the first four EGF-like repeats, suggesting a possible role for the N-terminus in ligand binding. In agreement with that, the immunofluorescence based study showed that the N-terminal part of Eater binds to the G- bacteria *Serratia marcescens*, the G+ *S.aureus* and the yeast *C.silvatica*. Interestingly, binding of Eater to *S.marcescens* could be competed out by scavenger receptor ligands, suggesting it to be a scavenger receptor-like protein. In S2 cells, *eater* down-regulation alone reduced phagocytosis of *E.coli* and *S.aureus* to 35 and 25% of wildtype levels respectively. Furthermore, larval hemocytes from an *eater* null line showed 5-fold reductions in *S.aureus* and 2-fold reductions in *S.marcescens* phagocytosis. The inefficient clearance of *S.marcescens* due to reduced phagocytosis resulted in increased bacterial load and an increased susceptibility to the bacteria following *S.marcescens* infection in adults. This phenotype demonstrates the importance of Eater's role in *S.marcescens* phagocytosis. In a combined RNAi knockdown study in S2 cells, less than

20% phagocytosis was observed for both *E.coli* and *S.aureus* when *eater*, *dSR-CI* and *PGRP-LC* (a peptidoglycan receptor) were down-regulated simultaneously. This redundancy in receptors is beneficial for host defense, if the microbe is able to override one recognition mechanism due a mutation in PAMPs.

Another EGF-domain containing transmembrane protein, Nimrod-CI (Nim-CI) was classified as a receptor by Ando and group using a biochemical approach (Kurucz et al., 2007). The authors initially characterized plasmatocyte-specific antibodies P1a and P1b (Kurucz et al., 2003). Immunoprecipitation from hemocyte proteins with a mixture of both antibodies led to isolation of the antigen P1. MALDI-TOF mass spectrometry analysis indicated that the protein was a novel single pass TM and the gene was named *nimrod-CI.* Down-regulation of *nim-CI* by RNAi in larval hemocytes led to a striking 3-fold decrease in *S.aureus* phagocytosis, almost comparable to RNAi of *eater*. Unlike Eater, Nim-CI did not appear to play a role in the binding of *S.aureus*. Nim-CI also did not affect *E.coli* phagocytosis. Transient expression of Nim-CI in S2 cells, which normally do not express the protein, resulted in an increase in both *S.aureus* and *E.coli* phagocytosis. It is possible that Nim-CI is playing a redundant role for *E.coli* phagocytosis. As Nim-CI did not affect the direct binding of *S.aureus* to hemocytes, its exact role in phagocytosis is not clear. It may serve as a coreceptor transducing the signal to activate phagocytosis. Since Eater and Nim-CI have almost similar effects on *S.aureus* phagocytosis, it is possible they work together in the same

pathway. Genetic and biochemical interactions studies between them might shed insight on their relationship and reveal the exact function of Nim-CI.

Draper, a Ced-1 homolog is found on hemocytes and serves as a receptor for apoptotic cell engulfment (Manaka et al., 2004). Following up on the finding that Croquemort, another apoptotic cell receptor also recognizes bacteria, Franc and group (Cuttell et al., 2008) examined the possibility of a similar role for Draper. They used pHrodo conjugated *E.coli* and *S.aureus* particles to examine phagocytosis. The pHrodo conjugate is sensitive to pH and its fluorescence increases with a decrease in pH. In Draper down-regulated S2 cells, fewer (~15% reduced) cells took up the bacteria. In adult phagocytosis assays, the *draper* mutant showed reduced pHrodo intensity for both *E.coli* and *S.aureus*. Since pHrodo is an indicator of phagosome maturation, their data suggest that Draper is involved in phagocytosis (S2 cell) and/or maturation (mutant). The role of Draper and the identity of its ligand were revealed when Shiratsuchi and group (Hashimoto et al., 2009) initiated a study from the microbe's perspective. The authors used larval hemocytes and screened various *S.aureus* mutants for an effect on phagocytosis and identified lipoteichoic acid (LTA) as an important component. The uptake of *S.aureus* mutants for LTA was reduced by 50-70%. Since literature on the Nimrod family of receptors suggested they play an important role in *S.aureus* phagocytosis, the authors examined whether LTA might be a ligand for one of these receptors. If so, the receptor mutant would not show a difference in phagocytosis between the parental *S.aureus*

and the mutant Δ*ltaS*. This was not the case for *eater* mutant or for *Nim-CI* downregulated in S2 cells but *draper* mutant flies phagocytosed the wildtype and mutant strains equally. Hence, genetic evidence indicated that Draper is the receptor for the *S.aureus* ligand LTA. Wall teichoic acid (WTA) was also identified as a component recognized for *S.aureus* phagocytosis. However the *S.aureus* parental and mutant for WTA (*tagO*) was not phagocytosed equally by *draper* null hemocytes, suggesting that Draper is not involved in WTA- dependent phagocytosis. The phagocytosis of *Bacillus subtilis* which also expresses LTA was similarly decreased in *draper* mutants. The limited role of Draper in *E.coli* phagocytosis as seen in the previous study was also reinforced. Draper seems to be the major receptor for LTA as *draper* null hemocytes did not bind to LTA in immunofluorescence studies*. draper* mutants exhibited susceptibility to *S.aureus*, thus phagocytosis mediated clearance plays a significant role during *S.aureus* infection. Simultaneous down-regulation of *draper* with either *eater* or *Nim-CI* further decreased *S.aureus* uptake, implying Draper and the other Nimrod family of receptors are involved in independent pathways for *S.aureus* uptake.

Peptidoglycan Recognition Protein (PGRPs): PGRPs are evolutionarily conserved proteins which bind to the bacterial peptidoglycan (PGN). Induction of both the Toll and Imd pathways in *Drosophila* rely on detection of the PGN by the PGRPs. Human have 4 PGRP homologues while Drosophila have 13 PGRP genes. Due to the presence of the N-acetylmuramyl-L-alanine amidase

domain, PGRPs are predicted to have amidase activity which can bind and cleave PGN (Mellroth et al., 2003). However, not all of them have the catalytic activity, due to lack of a critical cysteine residue. In a functional study involving an RNAi screen of S2 cells to isolate genes regulating phagocytosis, PGRP-LC was identified (Ramet et al., 2002). The S2 cells with down-regulated PGRP-LC, showed reduced binding of *E.coli* to the cells. Phagocytosis of *E.coli* was reduced by 20% while *S.aureus* phagocytosis was not affected. Furthermore, PGRP-LC mutants also had much lower antimicrobial peptide induction in response to E.coli. (Choe et al., 2002; Gottar et al., 2002; Ramet et al., 2002). Hence, along with being a receptor for phagocytosis, PGRP-LC also initiates signaling through the Imd pathway for AMP induction. On a cautionary note, the adult PGRP-LC mutant did not show any difference in *E.coli* phagocytosis in a separate study (Garver et al., 2006). Since the adult phagocytosis assay is not as quantitative as a hemocyte based assay, a subtle phenotype like 20% reduction could go undetected. Also, due to fewer receptors in S2 cells as compared to hemocytes, phenotypes do not get masked. Still, further mutant based studies are required to verify the role of PGRP-LC *in vivo*.

Another PGRP regulating phagocytosis was characterized by our group. A genetic screen carried out in adult flies identified PGRP SC-1a (*picky*) as a phagocytic receptor for *S.aureus* (Garver et al., 2006). Only 25% of *picky* adults were able to phagocytose any *S.aureus*, while 83% of the same mutant flies could phagocytose *E.coli*, suggesting that PGRP-SC1a is

specifically involved in *S.aureus* uptake. The mutant also showed increased susceptibity to *S.aureus,* possibly due to a defect in cellular response. The interplay between the catalytic property of PGRP-SC1a and its role as a phagocytic receptor was also examined. While expression of catalytic PGRP-SC1a in *picky* mutants could completely rescue *S.aureus* phagocytosis, the non-catalytic mutant of PGRP-SC1a showed a partial rescue. The inefficient rescue by the non-catalytic mutant was also observed for *S.aureus* clearance and survival analysis. Interestingly, if the *S.aureus* was left in an *S.aureus* conditioned media or free peptidoglycan was added to the resuspended *S.aureus*, the low efficiency of the non-catalytic PGRP was rescued. The authors suggested free peptidoglycan either generated by the action of PGRP-SC1a on *S.aureus* or added externally helps improve the efficiency of phagocytosis. Hence PGRP-SC1a act as a receptor, and due to its catalytic property generates more ligands to enhance the efficiency of the recognition process.

Down syndrome cell adhesion molecule (Dscam): Immunoglobulin (Ig) domain containing receptors are an essential component of the mammalian adaptive immune response. Somatic gene rearrangement provides extreme diversity to the receptors; hence potentially any antigen can be recognized. The *Drosophila Dscam* gene, initially characterized for its essential function in neuronal wiring, is also a member of the Ig superfamily. However the source of variability in this Ig family member is not somatic rearrangement, but

alternative splicing of *Dscam* which can potentially generate around 19000 different extracellular domains. The potential diversity in Dscam receptors led Schmucker and group to examine the expression and possible role of Dscam in hemocytes (Watson et al., 2005). Dscam protein could be detected in the larval hemocyte, hemolymph and *Drosophila* derived S2 cells. A soluble Dscam, possibly a proteolytic cleavage product was present in hemolymph and S2 cell conditioned media. The soluble Dscam could potentially recognize microbial ligands in the hemolymph. To examine the role of Dscam in phagocytosis, a transallelic combination of a hypomorph and an amorph was used. The larval hemocytes from the mutant showed approximately a 30% reduction in *E.coli* phagocytosis. Hemocyte-specific Dscam down-regulation also caused a similar reduction in phagocytosis confirming the likely origin and function of Dscam in the hemocytes. Addition of Dscam antibody to S2 cells reduced phagocytosis indicating that the extracellular domain or the secreted form plays an important role in the recognition of microbial ligand. Flow cytometry studies further confirmed binding of certain Dscam isoforms to live *E.coli*. Of the three isoforms studied, only two could bind *E.coli*. This left the question as to whether Dscam isoforms show binding specificity to certain microbes. Although, this has not been shown in *Drosophila*, it was beautifully addressed by Dimopoulos and group by examining the role of Dscam in cellular immunity of *A.gambiae* (Dong et al., 2006). A microarray study on the Sua5B cell line indicated that splice isoforms expressed in response to G- bacteria are different from those

induced by G+ bacteria. The induced isoforms also show binding specificity towards the respective microbe. The importance of this induction was corroborated by survival data in *A. gambiae* adults. While a general downregulation of Dscam increases susceptibility to both G+ and G- bacteria, downregulating the isoforms which are specifically induced by a G+ or G- microbe increases susceptibility to its respective inducer. Hence the Dscam isoforms show specificity in recognition and function.

The broad range of receptors identified signifies the importance that evolution has given to recognition of foreign matter. Different categories of receptors recognize different microbial ligands so the host can defend itself from a wide range of ever evolving pathogens. However, whether all of the receptors discussed above can function on their own or act only as co-receptors to facilitate the ligand attachment or signaling are questions that need to be answered.

C. Possible role for opsonins in Drosophila phagocytosis?

Opsonins are proteins which bind to a microbe and increase the efficiency of its phagocytosis. This process is called opsonization. In mammals, complement and antibodies act as opsonins. Drosophila has a family of four complement like thioester containing proteins: TepI, TepII, TepIII, TepIV. They show limited (28%) similarity to the conserved C3d region of the human complement protein C3. A phylogenetic analysis of thioester containing proteins indicated *Drosophila* (*dTEP*), *A.gambiae TEP-1* (*aTep1*), and *C.elegans TEP* (*cTEP*) , fall in the same clade, which is distinct from the

complement protein clade. Thus insect and worm Teps probably represent a primitive and distinct group from complement. In *Anopheles*, immunolocalization studies indicated Tep1 is expressed in hemocytes. Tep1 was also detected in hemolymph suggesting that it is secreted from hemocytes (Levashina et al., 2001). Interestingly, like complement, Tep1 gets denatured by heat, leading to fragmentation at the thioester motif. Also similar to mammals, a proteolytic cleavage product of Tep1 showed thioester motif-dependent binding to *E.coli* and *S.aureus*. The phagocytosis of *E.coli* by mosquito 5.1 cells was enhanced by the addition of conditioned media. This suggests a protein secreted from the 5.1 cells can increase the efficiency of phagocytosis of *E.coli*. Tep1 is induced in adult mosquitoes in response to *E.coli* but not *Micrococcus luteus*. Also, RNAi-induced downregulation of Tep1 in the 5.1 cell line led to decreased phagocytosis of G- *E.coli, Serratia marcescens* and *Salmonella typhimurium*, but had no effect on G+ *Bacillus subtilis*, *M.luteus* and *S.aureus.* The data suggested Tep1 might serve as an opsonin specific for G- bacteria in mosquitoes. The role of Teps in mosquitoes was validated as Teps were again identified as regulators of phagocytosis in a dsRNA screen carried out in mosquito adults (Moita et al., 2005). In accordance with the cell line data, Tep1 down-regulation reduced *E.coli* phagocytosis by 60%, but unlike the cell line observation, *S.aureus* phagocytosis was also reduced by 40%. It is possible that the discrepancy is due to differences in Tep1 expression pattern between the the *Anopheles* cell line and hemocytes. Additionally, Tep4 was also identified as a regulator of

both *E.coli* and *S.aureus* phagocytosis, while Tep3 was found to be important for only *E.coli* uptake.

In Drosophila there is limited literature on Teps. In an S2 cell screen to identify regulators of *Candida albicans* phagocytosis, Mcr was identified (Stroschein-Stevenson et al., 2006). Mcr is a member of the α2 macroglobulin/complement family of proteins. It is closely related to Teps in *Drosophila* but lacks the cysteine residue that is required for defining the thioester group of Teps. Downregulation of Mcr in S2 cells decreased phagocytosis of *C.albicans* specifically, while *E.coli* and *S.aureus* phagocytosis was unaffected. Mcr, like its Tep counterpart, is secreted. An S2 cell conditioned media could rescue the decreased *C.albicans* phagocytosis induced by downregulated Mcr expression. Biochemical studies confirmed binding of Mcr to *C.albicans*, indicating that Mcr might function as an opsonin. The same study also looked at the role of Teps in S2 cell phagocytosis. The role of TepI in *E.coli* phagocytosis could not be confirmed but TepII and TepIII were shown to have a role in *E.coli* and *S.aureus* phagocytosis respectively. In both cases, the reduction in phagocytosis was approximately 25%. Forward genetic studies might shed more light into the relative contribution of Teps in phagocytosis. Dscam is another protein suggested in the literature as having an opsonin-like role (Watson et al., 2005). A truncated version of the protein with no cytoplasmic C-terminal domain could be found in hemolymph. It is also also present in S2 cell

conditioned media. Since the N-terminus of Dscam can bind to *E.coli*, it is possible that Dscam can also function as an opsonin.

D. Adhesion regulates phagocytic uptake

Another player regulating phagocytosis via its role in adhesion is the family of Transmembrane 9 (TM9) / Nonspanin proteins. The TM9 family is characterized by the presence of a large variable extracellular N-terminal domain followed by nine putative transmembrane domains in their conserved C-termini. This family is conserved in the phagocytic amoeba *Dictyostelium* (Phg1), yeast, *Drosophila* (TM9SF), human (TM9SF) and plants. The TM9 family is divided into two subgroups. Phylogenetic analysis reveals that *Dictyostelium* Ph1b, *Drosophila* TM9SF3, and human TM9SF1 and TM9SF3 belong to subgroup I, while *Dictyostelium* Phg1a, *Drosophila* TM9SF2 and TM9SF4, and human TM9SF2 and TM9SF4 belong to subgroup II. In *Dictyostelium Phg1a* and *Phg1b* mutants had defects in phagocytosis. For *Phg1a* the defect was seen for a wide range of G- bacteria and for the G+ *Streptococcus bovis*, although the defect was more pronounced with G- bacteria. For *Phg1b*, only phagocytosis of G- bacteria was investigated. The defect was attributed to an inability to attach to the microbes. Interestingly, adhesion to hydrophilic versus hydrophobic otherwise inert substrates was differentially affected in the mutant. For instance adhesion to hydrophilic carboxylate group or amino group substituted beads was decreased but there was no difference in adhesion to hydrophobic latex beads. It is possible that the TM9 proteins regulate adhesion via control of the protein composition of

the phagocyte cell surface, as the *Phg* mutants showed an altered electrophoretic profile of the cell surface proteins (Cornillon et al., 2000; Benghezal et al., 2003).

A knock-out mutant of TM9SF4/Phg1a in *Drosophila* showed increased susceptibility to G- but not to G+ bacteria (Bergeret et al., 2008). The susceptible phenotype was found to be due to decreased phagocytosis. The *TM9SF4* mutant showed reduced phagocytosis of *E.coli* particles and latex beads, while phagocytosis of *S.aureus* particles was unaffected. Further RNAi analysis in S2 cells, validated the role of TM9SF4 in phagocytosis. The study also identified TM9SF2 as being redundant to TM9SF4. The former playing a role in *S.aureus* phagocytosissimilar to TM9SF4 in regulating phagocytosis. Upon examination of adhesion and spreading on glass, TM9SF4 mutant hemocytes displayed irregular spreading, along with a disrupted adhesion, indicating defective cell substrate contact. The actin cytoskeleton was also disorganized in the TM9SF4 mutant indicating that signaling downstream of recognition might be affected in the mutant. Hence the TM9 family proteins play an evolutionarily conserved role in adhesion to a substrate or microbe. Probably, one or more TM9 proteins can facilitate adhesion to microbe, as seen in the case of *S.aureus*, where either TM9SF2 or TM9SF4 were required for adhesion, while for *E.coli*, the function of TM9SF4 was essential.

E. Engulfment

Once a phagocytic cell has recognized and bound a microbe, actin cytoskeleton rearrangement is essential for the formation of the phagocytic

cup which engulfs the microbe into an intracellular compartment called the phagosome. In mammals, Fc receptor mediated phagocytosis is the best characterized. The zipper model for phagocytosis suggests that the phagosome is formed as the membrane and the cytoskeleton advances in a zipper-like manner over the microbial surface. This arrangement of cytoskeleton is preceded by signaling downstream of the engaged receptor. As a result, the small GTPases of the Ras superfamily like Rho GTPases, Cdc42 and Rac1 become activated. The expression of dominant negative forms of these GTPases results in inhibition of phagocytosis in mammalian cells. The phenotype is attributed to the inability to form a functional phagocytic cup (Cox et al., 1997; Massol et al., 1998). As expected, Cdc42 and the Arp 2/3 complex, a central regulator of actin cytoskeleton were identified in RNAi screens to identify regulators of phagocytosis in *Drosophila* (Agaisse et al., 2005; Philips et al., 2005).

During phagocytosis, the recruitment of the Rho GTPase to the membrane is essential. Rab GTPases regulate vesicular transport in a cell and have been proposed to regulate transport of Cdc42 and Rac1 in the cell. Kim and colleagues identified a Rab35 mutant in a forward screen for flies susceptible to *E.carotovora subsp. carotovora 15* infections. The mutant showed reduced phagocytosis of *E.coli* particles. Initiation of phagocytic uptake, requires formation of cell protrusions called lamellipodia and filopodia or commonly referred to as ruffles. Rab35 downregulated SL2 cells displayed distinctly reduced ruffles, suggesting vesicular transport of certain proteins is

required. Immunofluorescence studies with GFP- tagged Rab35 indicated cytoplasmic as well as plasma membrane localization. This is unique to Rab35, as other Rab GTPases are primarily found in cytoplasmic compartments. The Rho GTPases Cdc42, and Rac1 colocalize with Rab35. Epistatic analyses between Rho GTPases and constitutively active (CA) or dominant negative (DN) Rab35 indicated that Rab35 regulates ruffle formation through the Rho GTPases. An active Rab35 transports the RhoGTPases, possibly on a microtubule track to the plasma membrane where Rho GTPases control the actin polymerization required during phagocytosis (Shim et al., 2010).

The *S.aureus* receptor, Draper also signals through Rac GTPases. Rac1 and Rac2 have been shown to function downstream of Draper in a genetic interaction study (Hashimoto et al., 2009). Whereas loss of one copy of Draper, Rac1 or Rac2 had no effect on phagocytosis, simultaneous hetrozygous loss of each of them together led to a significant downregulation of phagocytosis. Thus, following recognition by Draper, there is a requirement for either Rac1 or Rac2 for uptake of the microbe.

In mammalian systems, the downstream effectors of Rac1 and RhoGTPase are WASP, N-WASP and WAVE of the Wiskott-Aldrich syndrome protein (WASP) family. These proteins bind to actin, profilin and the Arp2/3 complex and act at different stages of actin nucleation (Nakagawa et al., 2001). Drosophila D-SCAR, the homolog to WAVE was identified in a forward genetic screen as a regulator of *E.coli* and *S.aureus* phagocytosis (Pearson

et al., 2003). However, unlike its mammalian homolog, it is not concentrated in the phagocytic cup but is expressed throughout the cytoplasm. RNAi-induced downregulation of D-WASp in S2 cells also led to reduced phagocytosis of *S.aureus* bioparticles. Although it is known that these proteins regulate actin, the exact functions of D-SCAR and D-WASp are not well understood. Another actin regulatory protein, Profilin binds to free actin. The Profilin mutant, unlike D-SCAR and D-WASp mutants, shows increased phagocytosis of *E.coli* and *S.aureus,* possibly due to increased availability of free actin. Genetic interaction studies demonstrated that Profilin interacts with D-SCAR during phagocytosis (Pearson et al., 2003). Downregulation of Profilin also led to an increased phagocytosis of *Mycobacterium fortuitum* (Philips et al., 2005). Thus, regulation of actin cytoskeleton is a housekeeping component of phagocytosis, a defect in which leads to a general inability to take up any microbe.

F. Phagosome maturation

After internalization into a phagosome, the cargo of microbe or apoptotic cell has to be degraded. Interestingly, very different responses are generated for these different natured cargos. A microbial uptake should make the host ready for an immunological response, while uptake of an apoptotic cell should be immunologically silent. Still it has been found, so far, that all the phagocytic events follow the general paradigm of an endocytic pathway (Fig. 1-1). It is likely that cargo specific signaling in the endocytic compartments dictates the immunological response; the differences in maturation of an

apoptotic cell versus a microbe containing phagosome are an active area of investigation. During the receptor-mediated endocytic pathway, the cargo-containing endocytic vesicle fuses with the early/sorting endosome. The early endosome matures from a Rab5-positive early endosome stage to a Rab7-positive late endosome stage (Rink et al., 2005) and finally fuses with a lysosome. The presence of low pH in the lysosome activates hydrolytic enzymes like acidic proteases leading to degradation of the cargo.

RabGTPase tethers the vesicles to facilitate the fusion process (Vieira et al., 2002). In the active state, these GTPases decorate the endosome membrane and recruit their effectors, which help them carry out their function of regulating fusion processes as the maturation progresses. Other important proteins and protein complexes known to drive the maturation process are: phosphatidylinositol 3-kinase (PI 3-kinase), Endosomal sorting complex required for transport (ESCRT) complex and Vacuolar protein sorting-C (Vps-C) complex.

PI3-kinase - PI3-kinases are a family of enzymes that phosphorylate the phosphatidylinositol (PtdIns) at the 3rd position of the inositol ring. There are three classes of PI-3 kinases: Class I, II, III. Of those, only class III PI-3 kinases are conserved from lower eukaryotes to mammals. The mammalian homolog of Class III PI 3-kinase, Vps34, has been implicated in phagosome maturation (Vieira et al., 2001). Vps34 is associated with p150, a Vps15-like serine/threonine kinase.

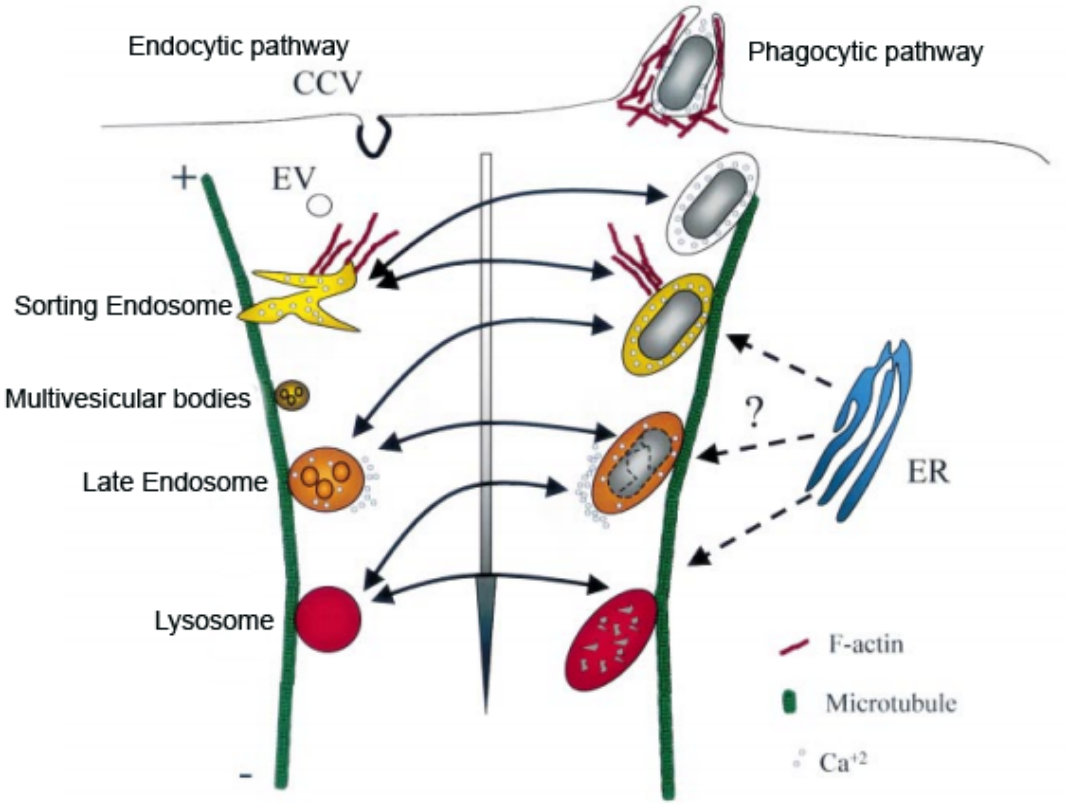


Figure 1-1. A diagram illustrating interactions between the endocytic and phagocytic pathway. The vesicles move towards the negative end of the microtubule. The arrow indicates the direction of maturation which is associated with increase in acidity of vesicles. (Vieira et al., 2002)

Vps15 contains an N-terminal myristoylation signal, and it regulates the membrane association and activity of Vps34 (Vieira et al., 2002). Vps34 generates phosphatidylinositol-3phosphate (PI-3P) which interacts with proteins containing the conserved FYVE (for conserved in **F**ab1, **Y**OTB, **V**ac1 and **E**EA1) domain. Early endosome antigen 1 (EEA1) is an effector of Rab5 and is required for fusion of the endocytic vesicle with the early endosome (Simonsen et al., 1998). The PI-3P generated by the PI-3kinase is required for stabilizing EEA1 on the early endosome membrane. Failure to do so leads to loss of EEA1 from the endosome and inhibition of phagosome maturation (Vieira et al., 2001).

In Drosophila, *ird1*, a Vps15 mutant, is susceptible to microbes like *E.coli* and *M.luteus* (Wu et al., 2007). Since the mutant has altered antimicrobial peptide (AMP) induction, part of the susceptibility could be accounted for by this immune defect. But phagosome maturation studies in this mutant are lacking. It is possible that deficient phagosome maturation, coupled with low AMP production could lead to increased susceptibility of the *ird1* mutant. Examining phagosome maturation in the *ird1* mutant can give an understanding of Vps15's role, which is relatively less understood compared to Vps34.

ESCRT- These proteins are required for formation of late endosomal structures known as multivesicular bodies (MVB). MVBs are formed when portions of the limiting membrane of the late endosome invaginates to form the intraluminal vesicles (ILV). The MVB pathway is important for turnover of

lipids and transmembrane (TM) proteins. The TM proteins that need to be degraded are targeted to the ILV. Sorting to the ILV is through ubiquitin tags found on the TM protein. These tags are recognized by the ESCRT complex which helps for efficient sorting into ILVs. However, the role of the ESCRT complex in phagosome maturation is not clear.

Perrimon and group reported that dVps28, an ESCRT I complex member in Drosophila is required for infection of S2 cells by pathogenic *M.fortuitum*, as characterized by decreased expression of a *Mycobacterium map*::GFP reporter (Agaisse et al., 2005). The reporter expression could be affected by two different means: degree of infection or phagosomal environment. An effect on the phagosomal environment could prove advantageous to a non-pathogen and may lead to its proliferation in the phagosome. Following this hypothesis, the same group further examined whether the altered phagosomal environment has any effect on the growth of non-pathogenic *M.smegmatis* (Philips et al., 2008). Downregulation of various ESCRT complex components like Vps4, Vps28 and Tsg101 in S2 cells led to intracellular growth of *M.smegmatis.* Additional studies on mammalian macrophage like RAW cells also showed significantly increased *M.smegmatis* cfu after downregulation of Tsg101. Hence, in the absence of the ESCRT complex, not only is the macrophage not able to clear the microbe, but the phagosome also allows bacterial growth, suggesting that the ESCRT complex plays an important role in phagosome maturation of microbe and clearance of pathogens. The ESCRT complex has also been shown to

play an important role in the pathogenesis of *Listeria monocytogenes*. *Listeria* evades maturation by escaping into the cytosol using two different proteins that it produces: a pore-forming cytolysin termed listeriolysin-O (LLO) and phospholipase C (PLC). An LLO-negative mutant cannot escape from the phagosome.

Portnoy and group screened S2 cells to identify genes that LLO might be targeting or where the absence of which would compensate for loss of LLO in *Listeria* (Cheng et al., 2005). The ESCRT complex (Bro1, Vps4, SNF7) and Vps-C (Vps16) complex proteins were identified. Absence of ESCRT complex may stall phagosome maturation, giving an LLO-negative *Listeria* enough time to escape using just PLC.

Vps-C complex- Vacuole protein sorting genes were identified initially by screens in yeast. The most severe phenotype mutants in terms of growth, trafficking and organelle morphology defects were grouped as Class C *vps* mutants. Thus Class C Vps proteins are required for multiple endolysosomal trafficking events. There are two Vps-C complexes: CORVET (class C core vacuole/endosome tethering) and HOPS (homotypic fusion and protein sorting). A HOPS complex member Vps16B has been implicated in phagosome maturation in *Drosophila* (Akbar et al., 2011). The hemocytes of the Vps16B mutant *full of bacteria* (*fob*) could never clear an infection from non-pathogenic *E.coli*. This results in increased bacterial load and susceptibility to *E.coli*. Immunofluorescence studies on hemocytes indicated that while recruitment of early and late endosomal markers Rab5 and Rab7

was normal, the lysosomal marker was not recruited onto the *E.coli* phagosome. Interestingly, fluid phase endocytosis of dextran was unaffected in the mutant. Thus Vps16B is required for fusion of microbe-containing late endosomes to lysosomes while it is not essential for maturation during fluid phase endocytosis. Drosophila has two Vps16 homologs Vps16A and Vps16B. Both of them are predicted to associate with two different Vps-C complex (Pulipparacharuvil et al., 2005). It is interesting to note that while downregulation of Vps16A is associated with defects in lysosomal delivery in eye discs, whereas Vps16B has no effect on endocytosis. The difference could be due to differential role of the proposed two different Vps-C complex in phagocytosis and endocytosis; Vps16A associated complex required during endocytosis and Vps16B associated complex required during phagocytosis.

The phagosome proteome

The phagosome is a very dynamic structure with its protein composition changing as the maturation progresses. The protein composition of the phagosome can give insight on both the known and unknown functions of the phagosome. Proteomic analyses of latex bead phagosomes in both the mammalian system and *Drosophila* have shed light on the complexity of this organelle (Garin et al., 2001; Stuart et al., 2007). SDS polyacrylamide gel electrophoresis followed by mass spectrometry identified 617 proteins associated with S2 cell latex bead phagosomes (Stuart et al., 2007). Upon

comparison to mammalian phagosomes, it was interesting to see that 70% of the mammalian phagosome proteins were also found on S2 cell phagosomes, indicating that the phagocytic machinery remains conserved during evolution.

Among the proteins identified on the S2 cell phagosome, of particular interest was the exocyst complex, as this complex was not known to be involved in phagocytosis. The exocyst is a complex of eight proteins (Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70, Exo84) that was initially identified in yeast for its requirement in secretion. Like Rab GTPase, the exocyst also helps in the tethering of vesicles, a prerequisite for fusion. Thus, in the absence of the exocyst complex, secretory vesicles accumulate at the plasma membrane, but there is no fusion with the plasma membrane, and hence no secretion. Ezekowitz and group identified six components of the exocyst on the phagosome. To ascertain the role of the exocyst in phagocytosis, *E.coli* and *S.aureus* internalization studies were carried out following silencing of individual exocyst components. Interestingly, Exo70 downregulation specifically reduced *E.coli* phagocytosis, while Sec3 downregulation reduced *S.aureus* phagocytosis. Other exocyst components like Sec8, 10 and 15 decreased both *E.coli* and *S.aureus* phagocytosis. The authors proposed a model wherein upon receptor ligand binding, a specific Rho GTPase gets activated. Since the Rho family is known to regulate the exocyst complex, depending on the type of Rho GTPase, either Sec3 or Exo70 gets activated on the phagocytic cup. Following activation, Sec3 or Exo70 will tether with the exocyst components Sec8, Sec10, Sec15 on the endosomal membrane,

leading to fusion of the phagosome with the endocytic vesicle. Hence, apart from regulating tethering during exocytosis, the exocyst might also help in the tethering of incoming phagosomes with early endosomes during phagocytosis. Thus, a previously unknown function of the exocyst was identified through the phagosome proteomics; however additional data is needed to support this role.

An emerging role for the phagosome is the release of the product of pathogen processing into the cytosol (Herskovits et al., 2007). Stuart and group identified an evolutionarily conserved solute carrier (SLC) transporter, SLC15, which could transport muramyl dipeptide (MDP) across the phagosomal membrane (Charriere et al., 2010). MDP is a peptidoglycan derived fragment, a potential byproduct of bacterial degradation. Mammals have Toll like receptors (TLRs) on the plasma membrane and the phagosomal membrane that recognize microbial PAMPs. Additionally, cytosolic NOD (nucleotide-binding oligomerization domain)-like receptors (NLRs) also recognize PAMPs. In macrophages, there is synergism between TLRs and NLRs for NF-kB activation in response to *S.aureus* (Charriere et al., 2010). But how cytosolic NLRs gain access to an extracellular microbe like *S.aureus* was an open question. The extensive Drosophila phagosome proteome database provided a promising candidate, Yin, a homolog to the human SLC15A family of transporters. PEPT1, another member of this family had also been implicated in MDP transport. Ectopic expression of both Yin and NOD2 in HEK293T cell led to increased NF-kB expression in the

presence of MDP. Similarly, the Yin homolog PEPT2 also led to increased NF-kB expression when expressed along with NOD2. This suggests in mammals, the phagosome might also release some degradation products into the cytosol through a phagosomal membrane transporter like Yin/PEPT2, which could then activate a cytosolic receptor like NLR. Activation of NLR along with TLR results in enhanced cytokine production which is beneficial to the host for limiting microbial proliferation. In Drosophila, PGRP-LE has been identified as a cytosolic receptor for diaminopimelic acid- (DAP) containing peptidoglycan. The recognition leads to activation of the Imd pathway. It will be interesting to see whether Yin transports the peptidoglycan that activates PGRP-LE in the hemocytes, or if such a collaboration is limited to the mammalian system.

Autophagy-role in immunity

After phagocytosis, an extracellular microbe gets eliminated during phagosome maturation, but an intracellular microbe like *Listeria* or *Mycobacteria* can subvert the maturation and either multiply in the phagosome itself, or multiply in the cytosol after exiting the phagosome. It is important for the infected cell to eliminate the intracellular pathogen, as the pathogen has escaped both the humoral response and phagosome maturation. Autophagy has been described as another cell based defense mechanism. Classically, autophagy is regarded as a highly conserved cellular mechanism for turnover of cytoplasmic organelles, proteins and nutrients

during starvation and cell death. Recently, autophagy has also been shown to be involved in defense against intracellular pathogens in mammals (Deretic, 2011).

G. Role of Autophagy in bacterial clearance

In Drosophila, autophagy helps in the elimination of the cytosolic intracellular pathogen L. monocytogenes (Yano et al., 2008). Autophagy induced clearance of Listeria follows recognition of Listeria's DAP-type peptidoglycan by the cytosolic receptor PGRP-LE. The hemocytes of PGRP-LE mutant larvae had increased loads of Listeria. This role for PGRP-LE can be distinguished from its function in the humoral response, as Imd pathway mutants (including the plasma membrane receptor PGRP-LC mutant) had wildtype levels of Listeria in their hemocytes. The Listeria mutant Δhly, which is incapable of entering the cytosol did not have increased cfu in the hemocytes of the PGRP-LE mutant. Induction of autophagy in the PGRP-LE mutant through rapamycin treatment or overexpression of Atg1, a regulator of autophagy, led to a decrease in Listeria levels in hemocytes. Hence autophagy-mediated clearance functions downstream of PGRP-LE. Listeria was found to localize in double membrane autophagosome-like structures in the cytoplasm by electron micrographs. Another interesting observation was that both lysine-type (G+) and DAP-type (G-) peptidoglycan were sufficient to induce autophagy in S2 cells with no requirement for the presence of a live microbe in the cytosol. However, unlike Lys-type peptidoglycan, DAP-type peptidoglycan could not induce autophagy in the absence of PGRP-LE,

suggesting that there is a separate, yet uncharacterized cytosolic receptor for the Lys-PGN in the cytosol.

H. Role of Rabs in phagosome maturation

RabGTPases, members of the Ras family of small GTPases, are essential for the regulation of intracellular membrane trafficking. They interact with SNAREs and motor protein which are principal mediators of specific membrane fusion and pericentriolar movement of vesicles during phagosome maturation. Thus RabGTPases serve as coordinators of vesicle trafficking (Stenmark, 2009).

What are Rab GTPases?

Rabs are members of the small monomeric Ras GTPase superfamily, identified initially in the **Ra**t **b**rain (Fig. 1-2) (Touchot et al., 1987). They switch between active GTP and inactive GDP bound states. The switch is regulated by Guanine Nucleotide Exchange Factors (GEF) which exchange GDP to GTP, and GTPase activating proteins (GAP) that catalyze the hydrolysis of GTP (Stenmark and Olkkonen, 2001). Experimental studies on Rabs have been carried out using dominant negative and constitutively active forms. There are conserved motifs in Rabs that are identified as phosphate/Mg2+ (PM) interacting loops. Mutation in these motifs leads to differential GTPase activity of Rabs. For instance, mutations in PM1 (Ser/Thr to Asn), G2 (Asn to Ile) or PM3 (Gln to Leu) leads to the expression of a dominant-negative GDP-

bound GTPase or a non-nucleotide associated form or a GTP-bound (constitutively-active) form of Rab GTPase respectively.

Some Rabs like Rab1a, Rab2a and Rab4a are expressed ubiquitously, while others like Rab3a (neurons) are tissue specific (Stenmark and Olkkonen, 2001). They are localized at the cytosolic face of intracellular membranes. Rabs also undergo membrane insertion and extraction cycles (Fig. 1-3). Membrane insertion requires post-translational modification of a C-terminal cysteine motif by the enzyme geranylgeranyl transferase. Newly synthesized Rab is recognized by the Rab escort protein (REP), which presents Rab to the enzyme for post-translational modification. After modification, REP delivers Rab to the membrane. A GDI displacement factor (GDF) then displaces REP from Rab, and this is followed by GEF-mediated activation of Rab. Activated Rab, recruits its effector and carries out its function. Upon GTP hydrolysis by GAP, Rab release from the membrane is catalyzed by the GDP dissociation inhibitor (GDI). GDI binds to Rab in the GDP bound form. Since GDI masks the geranylgeranyl anchor, Rab remains in cytosol. The GDI displacement factor (GDF) then helps in recruiting Rab again to the membrane (Seabra and Wasmeier, 2004).

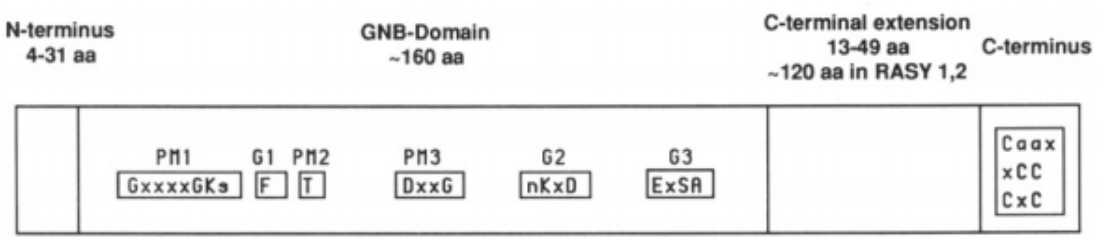


Figure 1-2. Conserved sequence elements in Ras-like proteins. GNB: Guanine nucleotide binding domain, PMn phosphate/ magnesium binding region, Gn guanine base binding region, a: aliphatic, X: any residue. (Valencia et al., 1991)

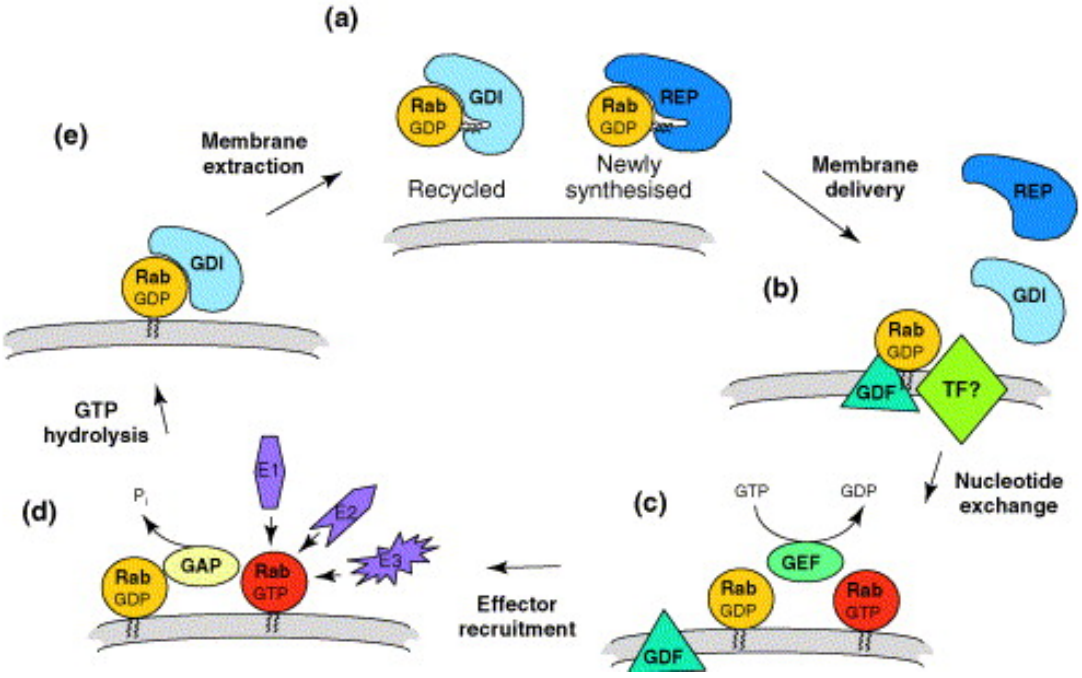


Figure 1-3. A schematic representation of the Rab recruitment, activation and extraction. REP or GDI proteins bring GDP bound Rab to the membrane, Replacement of REP or GDI by GDF exposes the membrane anchor of Rab, helping in membrane recruitment. Activation of Rab by GEF further helps in stabilizing the recruitment of Rab until GAP mediated hydrolysis of GTP. After which, Rabs are again extracted from membrane by GDI. (Seabra and Wasmeier, 2004)

Rab Effectors

The Rab effectors bind to the activated form of a specific Rab and mediate at least part of its downstream effect. Also, compartments associated with the endocytic pathway contain membrane domains for a specific Rab. Early endosomes contain distinct domains for Rab5 and Rab4, while recycling endosomes bear Rab4 and Rab11. Rab7 and Rab9 are found on late endosomes (Stenmark, 2009). It is proposed that the interaction between a Rab effector and Rab-GEF drives localization of Rab to its specific domain. For instance, the Rab5-GEF Rabex and its effector Rabaptin together localize Rab5 to early endosomes and endocytic vesicles. As Rab5 is recruited to the membrane, it gets activated by Rabex GEF activity. The activated Rab5 binds to the Rabaptin which in turn binds to Rabex 5 and stimulates its GEF activity (Stenmark, 2009). Thus Rab5 gets enriched on the membrane and then other effectors such as EEA1 and phosphatidylinositol-3-kinase (PI3K) are recruited and a membrane domain of Rab5 is formed.

Rab effectors in intracellular transport- During phagosome maturation there is a centripetal movement of phagosomes. Dyneins are microtubule motor proteins required for movement of cargo towards the minus end of the microtubule (towards the centrosome). **R**ab **i**nteracting **l**ysosomal **p**rotein (RILP), an effector for Rab7 has been demonstrated to be involved in mediating interaction of the phagosome with the motor protein dynein

(Harrison et al., 2003). Dynein regulates movement of phagosomes towards lysosomes as an essential step towards phagolysosome formation.

Rab effectors in tethering and membrane fusion- Tethering factors are another class of effector proteins. Tethering is a process that brings the compartments into close contact. It is a prerequisite for vesicle and target membrane fusion. Rab5 regulates homotypic early endosome fusion (Horiuchi et al., 1997). Phosphatidylinositol-3 kinases are effectors of Rab5 and their activity is essential for endosome fusion. PI-3Kinase regulates recruitment of the FYVE domain-containing Early endosome antigen 1 (EEA1) which is another Rab5 effector (Simonsen et al., 1998). The EEA1 dimer, a coiled coil tether is thought to bridge the two compartments, leading to their fusion (Callaghan et al., 1999). The multi-subunit tethering complexes, CORVET and HOPS are effectors of Rab5 and Rab7 respectively and play an essential role in phagosome maturation (Epp et al., 2011). The Rab effectors also interact with SNAREs, which are proteins essential for membrane fusion. Hence Rabs also regulate membrane fusion indirectly through effectors. For instance, the interaction of the Rab5 effector EEA1 and the tSNARE syntaxin13 is required for early endosome fusion (McBride et al., 1999).

Rabs change their state reversibly from an active GTP bound form to an inactive GDP bound form. Rab effectors only recognize the GTP bound form. The regions in the tertiary structure of Rabs that change in conformation in the GTP and GDP bound forms are called switch regions. There are two switch regions in Rabs called Switch I and II. A comparison of known

structures of Rabs (Sec4, Rab5, Ypt7, Rab11) reveal that their switch regions overlap significantly. Along with the two switch regions, a third region of contact between the effector and Rabs is the complementarity determining regions (CDRs) (Pfeffer, 2005). One Rab protein can interact with multiple effectors proteins associated with diverse functions. Also, effector proteins can be shared between different Rabs (Jordens et al., 2005).

Rab protein evolution

The Rab, Ras, Rho, Arf and Ran families are members of the Ras superfamily. A consensus phylogenetic tree based on the protein sequences from 19 species, sampling across *Animalia, Fungi, Plantae, Alveolates* groups indicate that the separation of the five GTPase families predated the expansion of eukaryotes. The Signal Recognition Particle Receptor (SRPRB) and Arf proteins are considered to be the founding members, which suggest that earlier functions of this family might be related to the regulation of membrane trafficking in a eukaryotic cell (Maria Rojas et al., 2012). RabGTPases form the largest family and are proposed to have evolved with a large number of genes to start in the Last Eukaryotic Common Ancestor (LECA), followed by loss or duplication in a lineage-specific manner (Brighouse et al., 2010). Fungi have relatively a low number of Rabs (8-12), which represents a massive loss from LECA (Brighouse et al., 2010).

Drosophila has around 33 Rabs (Zhang J. et al., 2007). Interestingly, the number of Rabs in *Drosophila* is comparable to *C. elegans* (29) an

organism with less than a thousand cells. More than 60 human Rab proteins and Rab- like members have been identified, suggesting a possible link between multicellularity and the number of Rabs (Schwartz SL et.al., 2007). However, contradicting this hypothesis is the fact that the protozoan parasite *Entamoeba histolytica* has over 90 Rab genes. Similarly, the protozoal parasite *Trichomonas vaginalis* has more than 300 Rab members, whereas other parasites like *Plasmodium falciparum* and *Toxoplasma gondii* have 11 and 15 Rabs respectively. Hence lineage-specific sculpting seems to be a major form of evolution for the Rab family (Brighouse et al., 2010).

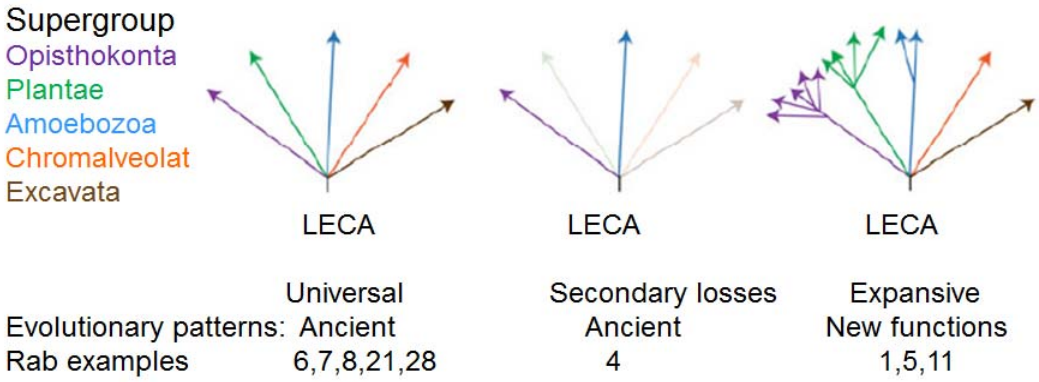


Figure 1-4. Rab protein evolution as inferred by genomics. The 5 hypothetical supergroups are denoted by colored arrows emanating from Last Eukaryotic Common Ancestor (LECA). The left most mode of evolution indicate origin of Rabs in or before LECA. The middle one suggests ancient origin accompanied by losses in some of the supergroups (as indicated by faint color). The right most mode of evolution is accompanied by expansion of paralogs associated with gain of function. (Brighouse et al., 2010)

III. Humoral response

Following infection, the humoral response leads to the release of antimicrobial peptides (AMPs) into the hemolymph, the blood equivalent for insects. The AMPs are mainly induced in the fat body, the insect equivalent of the liver, by the phylogenetically conserved Toll and Imd signaling pathways. These two pathways exhibit similarity to the mammalian TLR/IL-1R and Tumor necrosis factor-α (TNF-α) receptor signaling respectively.

In Drosophila, Toll signaling is induced by fungi, gram-positive (G+) bacteria and Drosophila X Virus (Lemaitre et al., 1996; Michel et al., 2001; Zambon et al., 2005). The Imd pathway on the other hand has been shown to be induced by gram negative (G-) bacteria (Lemaitre et al., 1995; Hedengren et al., 1999; Leulier et al., 2000). Expression of the AMPs, Drosomycin and Diptericin are typically used as indicators of an activated Toll and Imd pathway respectively. Additional studies in *Anopheles* mosquitoes suggest that Toll and Imd act in defense to the protozoan parasite *Plasmodium* (Cirimotich et al., 2010). Interestingly similar to the fungus and G+/G- specificity in *Drosophila*, the induction of Toll or Imd to protozoa seem to be parasite species dependent. For instance the *P.berghei* infection was controlled via the Toll pathway but *P. falciparum* infection was Toll pathway independent and was regulated by the Imd pathway. The Toll pathway has also been implicated in defense against dengue virus in mosquito *Aedes aegyptii* (Cirimotich et al., 2010).

A. Toll pathway

Drosophila Toll does not recognize pathogens directly; during an infection, fungi or G+ bacteria are recognized by secreted **p**attern **r**ecognition **r**eceptors (PRRs), such as **p**eptido**g**lycan **r**ecognition **p**rotein (PGRP) PGRP-SA (Michel et al., 2001), PGRP-SD (Bischoff et al., 2004) PGRP-SC1a (Garver et al., 2006), β glucan recognition proteins (GNBP) GNBP-1 (Gobert et al., 2003) and GNBP3 (Gottar et al., 2006) that lead to activation of the Toll pathway. The mutants for these PRRs show decreased survival upon G+ bacterial infection.

The recognition of the pathogens initiates a serine protease cascade which culminates in the activation of the Toll receptor ligand, Spatzle (Schneider et al., 1994; Weber et al., 2003; Shia et al., 2009). Serine proteases are also directly activated by fungi. A fungal virulence factor PR1 can directly activate the *Drosophila* serine protease persephone (Gottar et al., 2006). Persephone cleaves pro-Spatzle into activated Spatzle. Necrotic, a serine protease inhibitor, can inhibit persephone and thereby negatively regulate the Toll response (Levashina et al., 1999). Hence, fungal virulence factors can directly upregulate the Toll pathway. Active Spatzle binds to Toll as a dimer, leading to dimerization of the receptor. The adaptor proteins MyD88 and tube get recruited to the receptor along with the IRAK-like kinase pelle. (Horng and Medzhitov, 2001; Sun et al., 2002; Tauszig-Delamasure et al., 2002). Further downstream, Cactus, an IκB homolog gets phosphorylated and degraded by the proteasome, resulting in the release of the Rel

transcription factors Dif and Dorsal, which then move into the nucleus (Wu and Anderson, 1998), activating the transcription of AMP genes.

B. Imd pathway

Upon recognition of G- bacteria, the receptors PGRP-LC (Gottar et al., 2002) and PGRP-LE (Takehana et al., 2002) recruit the adaptor IMD (Choe et al., 2005). IMD in turn recruits *Drosophila* FADD homolog via homotypic death domain interaction (Naitza et al., 2002). FADD then interacts with the caspase DREDD, which is essential for the signaling downstream of IMD (Hu and Yang, 2000; Leulier et al., 2000). Following activation of signaling, IMD is cleaved in a DREDD dependent manner, exposing an IAP binding motif (Paquette et al., 2010), allowing IMD to associate with *Drosophila* inhibitor of apoptosis 2 protein (DIAP2). This results in IMD ubiquitination, following which an IKK signaling complex gets activated by IMD through Transforming Growth Factor Activated Kinase 1 (TAK1) (Silverman et al., 2003). IKK is composed of a kinase subunit Immune response deficient 5 (ird5 is an IKKβ homolog) and an accessory subunit Kenny (an IKKγ homolog), both of which are essential for signaling downstream of IMD (Rutschmann et al., 2000; Lu et al., 2001) . The activated IKK phosphorylates the Rel transcription factor Relish (Silverman et al., 2000; Erturk-Hasdemir et al., 2009). It has been proposed that DREDD might cleave phosphorylated Relish (Stoven et al., 2003). Cleavage of Relish releases the inhibitory domain (Rel-49) which

remains in cytoplasm while the amino terminal Rel domain (Rel-68) moves to the nucleus and activates transcription of Diptericin and other AMP genes.

IV. No hemocytes: The implications for the fly

Drosophila innate immunity has two components, cellular and humoral. In the absence of adaptive immunity, *Drosophila* makes an ideal host to examine the interaction between the two components *in vivo*. A thought provoking study by Rolf's group addresses the relative importance of these two defense responses towards *S.aureus* in the beetle, *Tenebrio molitori* (Haine et al., 2008). Almost 99.5% of an initial high dose of *S.aureus* was cleared within half an hour in the beetle. The induced humoral response measured by the antibacterial activity in the hemolymph was not active until 30 min and peaked at 24 hrs, but it lasted for almost 28 days after infection. This suggests that the initial near complete clearance was carried out by the constitutive response, an integral component of which is phagocytosis. The induced humoral response facilitated the complete clearance of the microbe. The authors hypothesized that the bacteria which survived the initial and humoral response-mediated clearance became more resistant to the immune response. In accordance to the hypothesis, *S.aureus* harvested from infected beetles at different timepoints after infection retained increased resistance and showed delayed clearance upon reinfection into a naïve beetle. Hence, the cellular response likely clears a majority of the bacteria during the initial

stage of infection. Whether the relative importance of the cellular and humoral response stands true against other pathogens requires further investigation.

Since hemocytes are the mediators of phagocytosis, examining the infection in a host with no hemocytes is an alternate approach to examine the relative importance of cellular immunity. The *domino* mutant in *Drosophila* has drastically reduced hemocytes, approximately 5% of wildtype. The mutation is in a gene regulating cell proliferation that results in the death of all differentiating hemocytes. The *domino* mutant dies in the third larval instar or prepupae stage. However, due to the pleiotropic nature of the mutation, the lethality could not be conclusively attributed to the absence of hemocytes. Examining the host-microbe interaction in larvae revealed that unlike wildtype, *domino* larvae have a wide range of microbes in the hemolymph (Braun et al., 1998). In experimental infection studies, *domino* larvae showed increased susceptibility to *B. bassiana* compared to a heterozygous *domino* control, while there was no difference in susceptibility towards *E.coli* and *M.luteus*. However in an *imd* mutant background, the *domino* mutation led to increased susceptibility to both *E.coli* and *M.luteus*. Hence both the humoral and cellular responses are required for clearance of *E.coli* and *M.luteus*.

The *Dif dorsal* double mutant is another fly strain that has been examined to understand the role of the hemocytes. The *Drosophila* Rel proteins, Dif, Dorsal and Relish are components of the Toll and Imd pathways. They also play an important role in the cellular immune response (Matova and Anderson, 2006). The Rel proteins Dif and Dorsal act as

negative regulator of apoptosis in hemocytes; the *Dif dorsal* double mutant had an approximately 8-fold increase in hemocyte apoptosis. The increased apoptosis led to a 10-fold reduction in the number of hemocytes in the *Dif dorsal* double mutant. The double mutant larvae have high loads of microbes in the hemolymph consistent, with results from *domino* mutants. Only about 3% of the double mutants could reach adulthood, but if reared in aseptic conditions, the survival to adulthood was increased by 10-fold. Hence, lack of a constitutive cellular response due to the absence of hemocytes, led to high bacterial loads in the larvae, causing larval mortality.

Since mutations are sometimes associated with pleiotropic effects, alternative for assessing the role of hemocytes is to functionally disable or potentially remove them from the system. In Drosophila, the initial study with functionally disabled hemocytes was carried out by Schneider and group (Elrod-Erickson et al., 2000). Injecting flies with beads led to uptake of the beads by hemocytes, preventing further phagocytosis in the fly. A bead saturated hemocyte presumably could be stalled at a stage of maturation, inhibiting further uptake. Limiting the cellular response by injection of beads exacerbated the susceptibility of a humoral response mutant towards non-pathogenic *E.coli*. For instance in *E.coli* survival studies, the bead-injected *imd* mutant attained 50% mortality, 48 hours earlier than the uninjected counterpart. The cellular and humoral responses thus work synergistically for defense to *E.coli*.

Another study examined the role of the cellular response towards the G+ bacteria, *Micrococcus luteus, Enterococcus faecalis* and *Staphylococcus aureus* (Nehme et al., 2011). The bead-injected flies showed increased susceptibility to all three G+ pathogens. Interestingly, an enhanced humoral response could compensate for the lack of a cellular response in a pathogen-dependent manner. In survival studies, ubiquitous expression of the AMP Defensin, or constitutive expression of the Toll pathway could compensate for the lack of a cellular response in defense against *M.luteus* and *E.faecalis* respectively. A similar rescue was not observed in response to *S.aureus* infection, confirming the pivotal role of the cellular response in defense towards *S.aureus*.

Hemocyte cell ablation studies have also given similar insights into the pathogenesis of different microbes. Genetic cell ablation has been carried out by expressing pro-apoptotic genes specifically in hemocytes. It should be noted the extent of hemocyte ablation is dependent on the timing and level of expression from the hemocyte driver; thus some hemocytes might escape apoptosis. Leulier and group observed with the embryonic hemocyte-specific driver serpent, only 6% of embryos reach the 1st instar larval stage, suggesting hemocytes are important for embryonic development (Defaye et al., 2009). A larval and adult stage hemocyte driver hemolectin led to a 74% decrease in larval circulating hemocytes. About 45% of the hemocyte-ablated larvae could survive to adulthood. Similar to the *Difdorsal* mutants, germ free media increased survival to adulthood. This observation is consistent with the

independent hemocyte ablation studies by the groups of Royet and Ligoxygakis (Charroux and Royet, 2009; Shia et al., 2009).

The survival studies following systemic infection compared the effect of loss of the humoral response versus the cellular response for different G+ and G- microbes (Table 1-2). While hemocyte ablation increased susceptibility towards *Erwinia carotovara carotovara 15* (G-), *E.coli* (G-), *Salmonella typhimurium* (G-), *P.entomophila* (G-), *E.faecalis* (G+), *S.aureus* (G+) and *C.albicans* (fungi), it did not have any effect against *Bacillus subtilis* (G+ with a DAP type peptidoglycan), *Staphylococcus saprophyticus* (G+) or *Streptococcus agalactiae* (G+).

A defect in the humoral response also had a more pronounced effect as compared to the cellular response in defense against *Ecc15, E.coli, P.entomophila, E.faecalis, B.subtilis, S.saprophyticus, S.galactiae* and *C.albicans* infection (Charroux and Royet, 2009; Defaye et al., 2009). For *S.aureus* and *S.typhimurium,* both hemocyte ablation and humoral response mutants showed comparable susceptibility, further reinforcing the role of the cellular response towards *S.aureus* (Defaye et al., 2009). Hemocyte ablation also increased susceptibility of the larvae to *E.coli, E.carotovora, M.luteus* (Shia et al., 2009). While wildtype larvae could completely clear a hemolymph infection within 2-3 h, hemocyte-ablated larvae could not clear an infection by that time and a rapid increase in bacterial titers were observed after 5 h (Shia et al., 2009).

Comparable roles for cellular response and humoral response	**Minor role of cellular response and major role of humoral response**	**No detectable role of cellular response**
S.aureus	*Ecc15*	*Bacillus subtilis*
S.typhimurium	*E.coli*	*S.saprophyticus*
	P.entomophila	*Streptococcus agalactiae*
	E.faecalis	
	C.albicans	
	M.luteus	

Table 1-1. Relative contribution of the cellular and humoral response against immune response to various pathogens. (Charroux and Royet, 2009; Defaye et al., 2009; Nehme et al., 2011)

It can be concluded hemocytes are essential for defense against opportunistic pathogens, as an absence of hemocytes led to low survival to adulthood, which can be rescued by a relatively sterile environment. Furthermore, initial control of infection by phagocytosis is important against almost all pathogens. The relative importance of the cellular and the humoral responses varies with the pathogen and is probably a result of the evolution of the host-pathogen interaction. A noteworthy observation is the bias of cellular or humoral immune response towards a certain pathogen is not based on the nature of their peptidoglycan (G+/G-). Potentially the bias could be dependent on the nature of the microbe associated pathogenesis.

V. Immunological memory in flies

The hallmark of innate immunity is recognition of broadly conserved patterns of pathogens. It is induced upon challenge from a microbe and there is no difference in the lag time or the extent of induction between the initial and subsequent challenge. Adaptive immunity found in mammals has the advantage of enhanced and faster induction upon repeated exposure. This enhanced induction is very specific to an antigen and hence to a pathogen.

An interesting study by Schneider and group, challenges these strict definitions of innate versus adaptive, as the group reported that an initial priming of the flies with *Streptococcus pneumoniae* helps the flies resist a repeated exposure against the same (Pham et al., 2007). An initial exposure

to either dead or lower CFU of *S.pneumoniae* decreased the susceptibility of the fly to a higher dose of *S.pneumoniae*. Later the mean survival of the fly after the second exposure was increased by almost 100% when compared to control. The effect of priming starts within 24 hrs. A similar protective effect was also seen against the natural fungal pathogen *B.bassiana*. However, prior infection with other pathogens such as *L.monocytogenes*, *M. marinum*, *Salmonella typhimurium* did not show similar protective effects upon reinfection. Importantly, prior exposure to *S.pneumoniae* did not protect against any other pathogen but *Streptococcus* itself, thus addressing the concern of specificity.

The protective response was also examined in the Toll pathway mutant *PGRP-SA*^{seml} and Imd pathway mutant *imd*^{10191}. While the imd pathway mutant retained a protective response, the Toll pathway mutant failed to elicit the protective effect of priming, indicating that the Toll pathway is required for priming. However the possibility that the receptor PGRP-SA is involved in priming through yet an uncharacterized pathway distinct from Toll cannot be excluded. Thus examining other Toll pathway mutants could validate role of Toll pathway in priming-induced protection. The role of AMPs in inducing the protection was excluded, as most of the *Streptococcus*-induced AMPs are down-regulated within a week, while the protective response was still observed after that time. In order to examine the role of hemocytes in the protective response, the hemocytes were functionally disabled by saturating them with beads. The bead-saturated hemocytes have

defects in phagocytosis (Elrod-Erickson et al., 2000). The discovery of hemocytes as a prinicipal mediator emerged, as the flies with bead saturated hemocytes were unable to evoke a protective effect after priming. Hence, in contradiction to general features of innate immunity, *Drosophila* displays a blood cell dependent defense response which has specificity and memory, the defining features of adaptive immunity. Since host pathogen interactions play an important role in the evolution of immunity, examining more natural pathogens for similar protective effects will give more insight on this intriguing adaptive feature of innate immunity.

The presence of innate immune memory has also been demonstrated by the Barillas-Mury group in *Anopheles gambiae* against its natural protozoan parasites, *Plasmodium berghei* and *P.falciparum* (Rodrigues, 2010). An earlier infection by *P. berghei* limited infection upon a second exposure to the same, and the protective effect was seen as early as 7 days post infection to 14 days post infection. Similar protection was seen against *P.falciparum* as well. The presence of gut bacteria was essential for the memory, as removal of gut bacteria either during the first or second infection inhibited the protective effect of priming. *Plasmodium* infection in the presence of gut bacteria increased hemocyte- associated gene expression in the midgut tissue, suggesting recruitment of hemocytes to the midgut. The infection also induced differentiation of prohemocytes into granulocytes. An important observation was that along with limiting *Plasmodium* infection upon re-exposure, a *Plasmodium*-challenged mosquito also had reduced midgut-

associated bacteria. The authors hypothesized that the mosquito immune system gets stimulated as the midgut associated microbes come in contact with the breached gut barriers induced by *Plasmodium* ookinetes during the first infection. This results in hemocyte differentiation into granulocytes. Upon re-exposure to ookinetes, a more effective antibacterial response is mounted, resulting in a collateral control of Plasmodium infection. A cell free hemolymph transfer from an infected fly could induce differentiation and protection against a second challenge in a naïve fly. The authors speculated on the presence of a signaling molecule induced in response to *Plasmodium* infection that regulates hemocyte differentiation. The protection seen in *Anopheles* has an enhanced secondary immune response, a feature of adaptive immunity but it lacks specificity. In *Drosophila,* the authors observed specificity in protection to bacteria but the extent of protection to other species of the same genus needs more study. It could be speculated that the adaptive aspect of invertebrate immunity is a component of innate immunity that got masked or lost in vertebrates upon the development of adaptive immunity.

VI. Are we talking? Signaling from hemocytes

Cytokines are the signals released from mammalian phagocytic cells in an acute phase response. These signals can then act on target cells at a distance to stimulate an appropriate response. In Drosophila, hemocytes secrete two cytokine-like molecules: Upd3, a ligand for the JAK-STAT

pathway and Spatzle, the ligand for the Toll pathway. The cytokine Upd3 is induced upon infection in hemocytes and is required for the synthesis of the acute phase protein TotA in the fat body (Agaisse et al., 2003). The fat body is also the main source of AMPs as it contributes 70-80% towards the total AMP production. Whether signaling from the hemocytes is essential for AMP production in the fat body is still open to debate as there is evidence both in support and against it.

Hemocytes form the initial line of defense once the epithelial barrier is breached. Phagocytosis takes place within an hour after the initial contact with a microbe, so it is possible that a signal from the hemocyte initiates and amplifies the Toll and Imd signaling in the fat body, which occurs later. *psidin*, a phagosome maturation deficient mutant showed no induction of the antimicrobial peptide (AMP) Defensin, in response to either G+ or G- infection, while other antimicrobial peptides were not affected. The authors also observed a similar phenotype for the hemocyte lacking *domino* mutant and phagocytic receptor *eater* mutants. The authors hypothesized that phagosome maturation might regulate the humoral response via an immunostimulatory signal. The basal level of *defensin* expression is low, making it a good indicator for changes in AMP expression (Brennan et al., 2007). In accordance with the hypothesis, hemocyte-specific expression of psidin rescued the Defensin induction in the *psidin* mutant.

If there is signaling from the hemocytes, then identification of the signaling molecule is the next critical step. In a hemocyte ablation study,

Ligoxygakis and group (Shia et al., 2009) observed reduced *diptericin* and *drosomycin* induction in larvae. In an effort to identify the signal, authors looked at *spatzle* mutants. Spatzle is the ligand for the Toll pathway in both dorsal-ventral embryonic patterning (Schneider et al., 1994) and the immune response (Weber et al., 2003) and its expression is increased in the hemocytes upon infection. *spatzle* mutants showed defective *drosomycin* induction but normal *diptericin* induction. Ectopic expression of *spatzle* in the hemocytes could rescue *drosomycin* induction in the *spatzle* mutant. Interestingly, downregulation of *spatzle* in the fat body had no effect on *drosomycin* induction, possibly because *spatzle* expression in the hemocyte is sufficient for AMP induction.

However, the signal hypothesis is not supported by other hemocyte ablation studies. The hemocyte ablated adults showed no difference in expression of *diptericin* and *attacin* in response to *E.coli* or *defensin* and *drosomycin* in response to *M.luteus* respectively (Charroux and Royet, 2009). Leulier and group also observed a comparable induction of *IM1* and *defensin* in the hemocyte-ablated flies and their respective controls in response to *S.aureus*, and of *diptericin* and *defensin* in response to *S.typhimurium* (Defaye et al., 2009). Similarly the bead-injected flies showed comparable induction of *drosomycin* and *defensin* in response to *M.luteus*, *E.faecalis*, *S.aureus* and *M.luteus* respectively (Nehme et al., 2011). The discrepancies could be a result of the nature of the different experiments. The extent of hemocyte ablation can be assessed in larvae by counting the circulating

hemocytes. But adults mostly have only sessile hemocytes and the only way to evaluate the presence of hemocytes is through a hemocyte-specific reporter. Not all embryonic and postembryonic hemocytes are regulated by the same driver. Since the same driver was used to drive expression of apoptotic genes and the reporter in the hemocytes, it is possible in adults, certain populations of hemocytes escaped apoptosis and were still functional. Similarly bead-injected flies might have just enough functional hemocytes to send the signal. It is also possible that the initial process of bead phagocytosis itself leads to expression of a 'signal' from the hemocytes. Further investigations might give a more definitive answer.

In conclusion, cellular response is an essential component of *Drosophila* response potentially regulating diverse processes as immunological memory and humoral response. The process that follows phagocytic uptake leads to clearance of microbes and can also regulate gene expression in the hemocyte. Since relatively less is understood about this process, we examined the process of phagosome maturation in *Drosophila*. We also try to address the possibility of a cross-talk between cellular and humoral response.

Chapter 2

A screen to identify RabGTPases regulating phagosome maturation

Abstract

Phagocytosis involves an interaction between the phagosome and the endocytic machinery. RabGTPases, essential components of the membrane trafficking machinery are known to regulate both phagocytic uptake and maturation. Since intracellular pathogens can subvert the process of phagosome maturation by targeting RabGTPases, understanding their role is essential to gain insight into the pathogenesis of intracellular microbes. There are approximately 33 RabGTPases in *Drosophila* and proteomics data indicate that 12 of them are associated with the *Drosophila* S2 cell phagosome (Stuart et al., 2007). Using an *in vivo* phagosome maturation screen we identify some candidate RabGTPases as positive regulators of the phagocytosis/ phagosome maturation process. The screen identified Rab2 which is known to regulate apoptotic cell phagosome maturation in *C.elegans*. Another candidate Rab14 is essential for *M.tuberculosis* pathogenesis. Rab6 and Rab18, two regulators of the retrograde pathway were also identified as candidates. This points towards a potential involvement of the retrograde pathway during phagosome maturation.

Introduction

An incoming phagocytic vesicle fuses with the early endosome ─ this sort of fusion is referred to as heterotypic fusion. The sorting endosomes also fuse with each other by a process referred to as homotypic endosome fusion. Rab5 has been implicated in both, homotypic and heterotypic endosome fusions (Somsel Rodman and Wandinger-Ness, 2000). Early phagosomes rapidly gain Rab5 (Desjardins et al., 1994a) and expression of a dominant negative allele of Rab5 leads to arrest of phagosome maturation (Scott et al., 2003). Rab5 has also been shown to regulate transient fusion followed by separation also called as 'kiss and run' fusion between the phagosome and early endosome. As a result, a constitutively active Rab5 leads to the appearance of giant phagosomes (Duclos et al., 2000). As maturation progresses, the Rab5 domain is disassembled and the endosome acquires Rab7 (Rink et al., 2005).

A phagosome also acquires Rab7 (Desjardins et al., 1994a), although not shown, it is likely that phagosomes acquire Rab7 in a similar manner as endosomes. The recruitment of Rab7 onto phagosomes is required for the subsequent fusion with late endosomes and lysosomes in the RAW 264.7 macrophage cell line (Harrison et al., 2003). Rab7 has also been shown to regulate phagosome maturation in the unicellular amoeba *Dictyostelium*.

Due to their role in membrane trafficking, RabGTPases are often targeted by intracellular pathogens in order to avoid the lysosomal killing process (Brumell and Scidmore, 2007). To avoid fusion with the lysosomes,

intracellular pathogens actively arrest maturation at either early or late endosomal steps. In mammalian cell culture studies, *Mycobacteria bovis*-containing phagosomes recruit Rab5, but do not mature into Rab7-positive compartments. Thus *M.bovis* actively prevents fusion with the late endosomal compartment (Via et al., 1997). Also, the mycobacterial cell wall component lipoarabinomannan, and the enzyme SapM inhibit accumulation of the Rab5 effector, EEA1 onto the endosomal membrane (Fratti et al., 2001; Vergne et al., 2005). Live *Mycobacteria* modify their phagosomes by retaining early endosomal RabGTPases, Rab14 and Rab22a for longer durations, presumably to continue fusion with early instead of late endosomes (Kyei et al., 2006; Roberts et al., 2006). Other intracellular pathogens like *Helicobacter pylori* recruit and retain Rab7 but still prevent fusion with lysosomes (Terebiznik et al., 2006). The *Salmonella enterica* serovar typhimurium containing vacuole (SCV) is also arrested at a Rab7-positive late endosomal stage. Like *Mycobacteria*, *Salmonella* actively modulates the Rab GTPases associated with its phagosome (Smith et al., 2007).

In order to understand the pathogenesis of intracellular microbes, it is essential to identify the RabGTPases that regulate phagosome maturation. We carried out an *in vivo* phagosome maturation screen using dominant-negative (DN) mutants of RabGTPases and identified Rab2, Rab6, Rab14 and Rab18 as potential positive regulators of phagosome maturation.

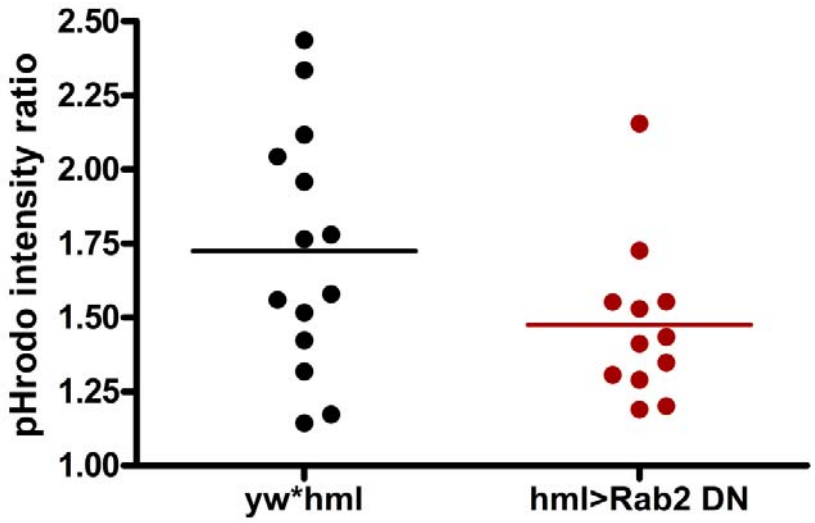
Results

In an attempt to identify other RabGTPases which might regulate phagosome maturation, we did a screen of the RabGTPases associated with latex bead phagosomes (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 fly stocks expressing dominant negative mutants of 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.* Unfortunately, we were not able to present data for the Rab5, Rab7 and Rab35DN lines. In our hands, the Rab5DN line does not show any increased susceptibility to *S.aureus* in survival assays or any defects in maturation. Since we have not sequenced the transgene, it is possible there is something wrong with the Rab5DN construct/transgenic line as these results are inconsistent with Rab5's predicted function in phagosome maturation. For Rab7 and Rab35 we were not able to do the experiment due to the unavailability of flies.

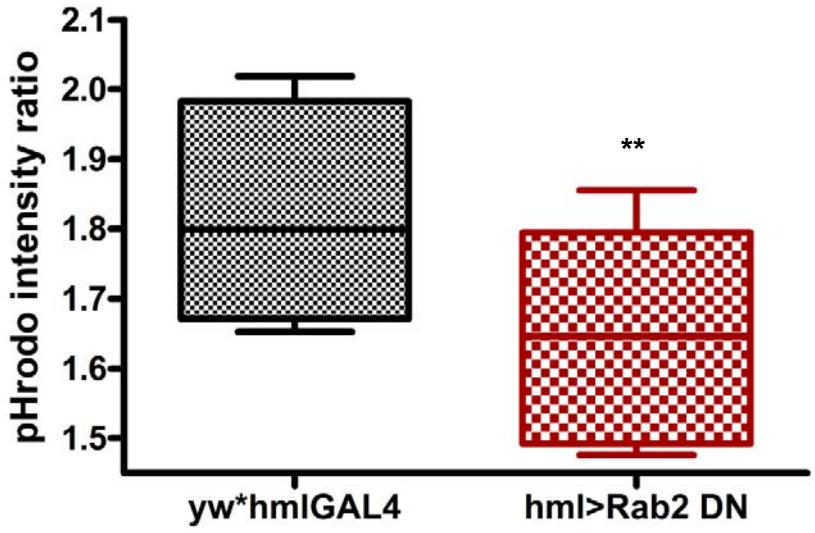
Rab2, Rab6 and Rab18 (Fig. 2A) were identified as potential regulators of phagocytic uptake and/or phagosome maturation in the screen. Expression of dominant negative mutants of Rab1, Rab4, Rab8, Rab10 and Rab11 did not affect phagosome maturation (Fig. 2B). Earlier RNAi-based studies (Shim et al., 2010) found no effect on phagocytic uptake of *E.coli*

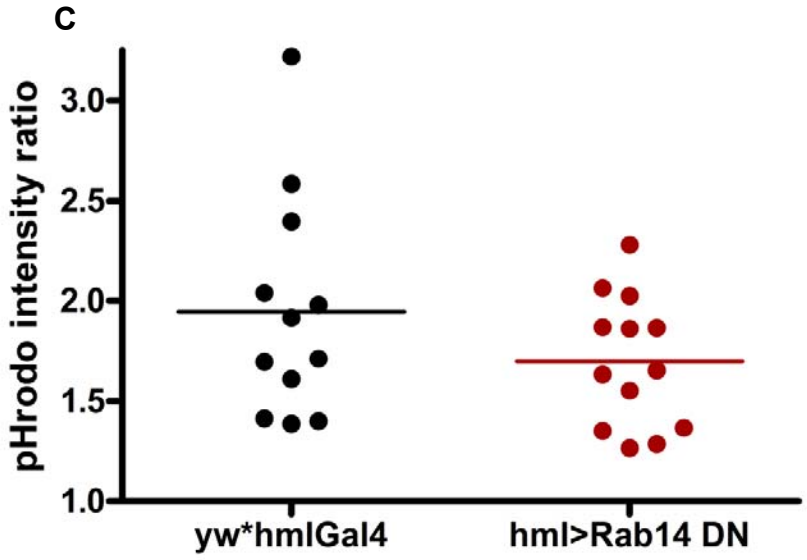
particles by S2 cells, after downregulation of any of the Rabs that we tested. This suggests that the demonstrated decreased pHrodo intensity could be due to defects in maturation. However, there might be differences in the regulation of uptake of *E.coli* compared to *S.aureus.* Hence *in vivo* uptake studies are required for validation.

A


pHrodo intensity ratio
2.50
2.25
2.00
1.75
1.50
1.25
1.00
yw*hml
hml>Rab2 DN

B


pHrodo intensity ratio
2.1
2.0
1.9
1.8
1.7
1.6
1.5
**
yw*hmlGAL4
hml>Rab2 DN


C
pHrodo intensity ratio
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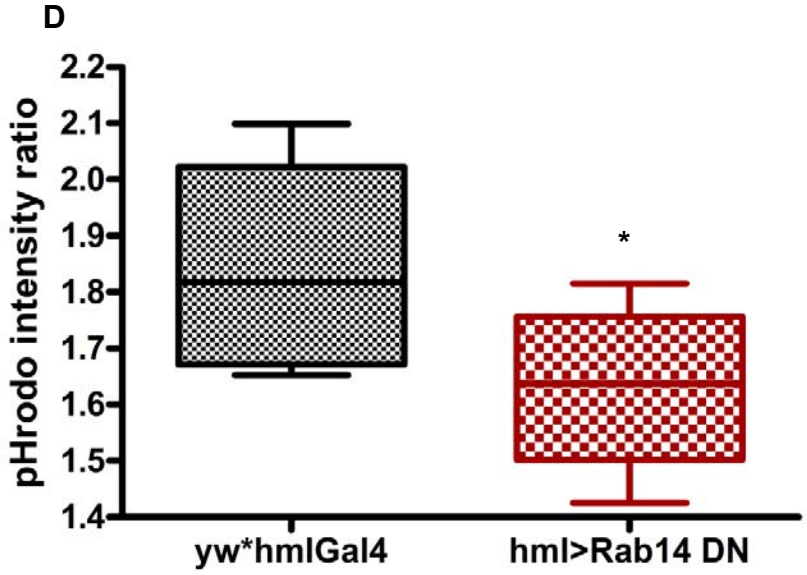
2.5
2.0
1.5
1.0
yw*hmlGal4
hml>Rab14 DN


D
pHrodo intensity ratio
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2.1
2.0
1.9
1.8
1.7
1.6
1.5
1.4
*
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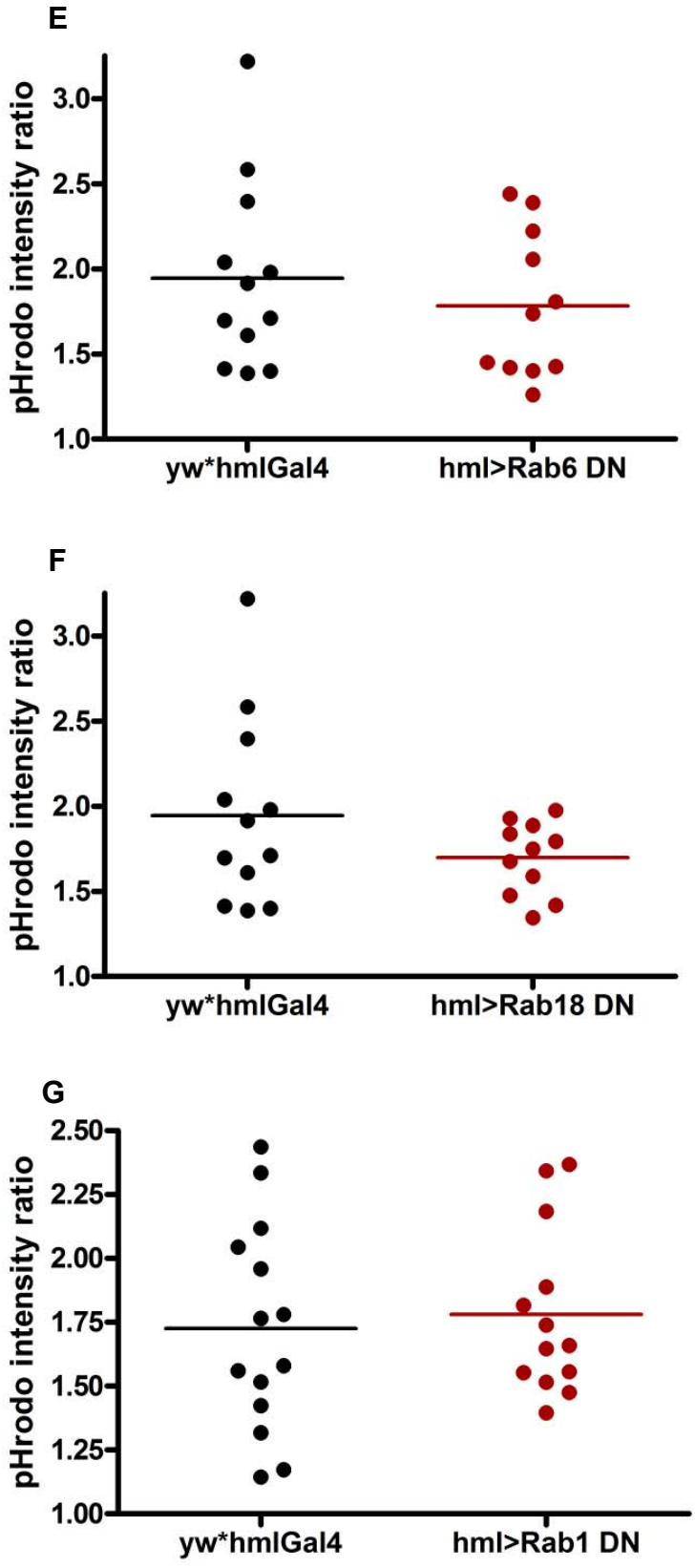
hml>Rab14 DN


E
pHrodo intensity ratio
3.0
2.5
2.0
1.5
1.0
yw*hmlGal4
hml>Rab6 DN
F
pHrodo intensity ratio
3.0
2.5
2.0
1.5
1.0
yw*hmlGal4
hml>Rab18 DN
G
pHrodo intensity ratio
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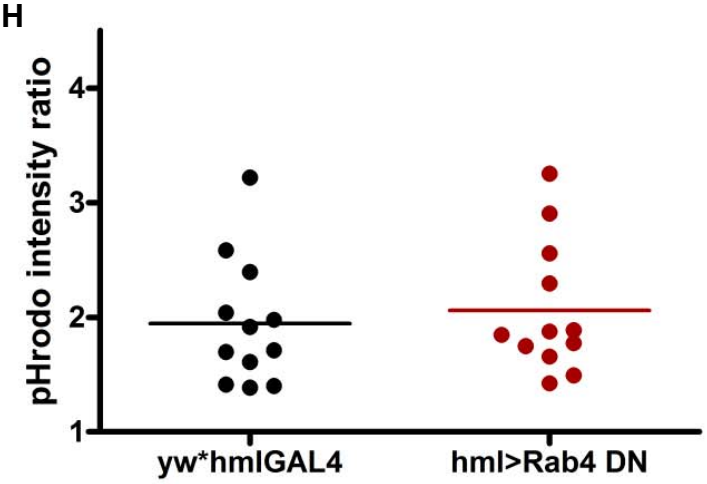
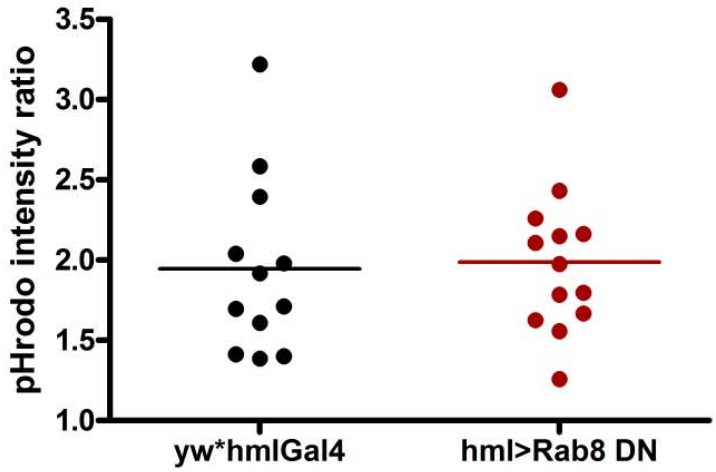
2.00
1.75
1.50
1.25
1.00
yw*hmlGal4
hml>Rab1 DN

H


pHrodo intensity ratio
4
3
2
1
yw*hmlGAL4
hml>Rab4 DN

I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hml>Rab8 DN

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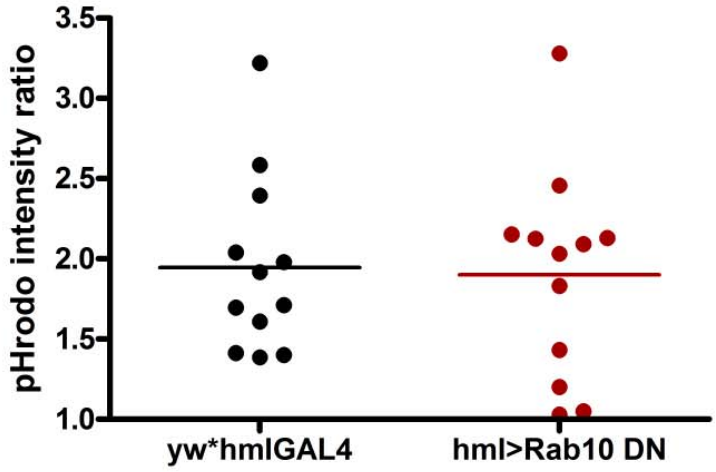
pHrodo intensity ratio
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yw*hmlGAL4
hml>Rab10 DN

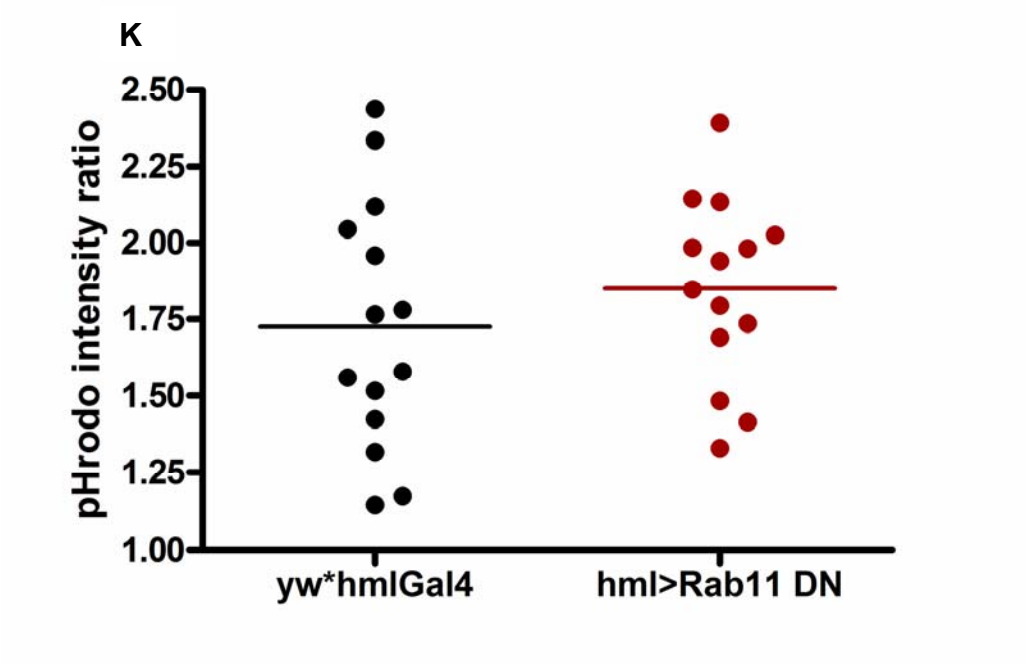

Figure 2-1. Identification of candidate RabGTPases that might regulate phagocytic uptake and/or phagosome maturation Adult flies were injected with pHrodo *S.aureus* and imaged after 60 min. The DN lines were crossed to hmlGAL4. The parental line of the dominant-negative expressing line (yw) outcrossed to the hemolectin driver line was used as the control. Representative dot plots for: (A) Rab2, n=6 (C) Rab14, n=4 (E) Rab6, n=2 and (F) Rab18, n=2 (G) Rab1, n=5 (H) Rab4, n=3 (I) Rab8, n=5 (J) Rab10, n=3 and (K) Rab11, n=2 are shown. Box and whisker plots for (B) Rab2 and (D) Rab14 have been plotted with n=6 and 4 for Rab2 and Rab14 respectively. n represents the number of experiments for individual RabGTPases. * $p < 0.05$ ** $p < 0.01$.

Discussion

Rab2 has been shown to regulate apoptotic cell degradation in *C.elegans* (Mangahas et al., 2008). Since microbial and apoptotic cell degradation display strong similarities (Kinchen and Ravichandran, 2008), Rab2 might also play an essential role in microbial phagosome maturation. If so, it will be interesting to examine what stage of phagosome maturation is regulated by Rab2 and whether it functions upstream or downstream to Rab14. Rab2 can potentially work with Rab14 to recruit Rab7 or it can work with Rab7 to facilitate fusion with lysosomes.

Another candidate, Rab6 is required for retrograde (early endosomes and Golgi to the endoplasmic reticulum) (Girod et al., 1999; Mallard et al., 2002) and anterograde (Golgi to plasma membrane) (Grigoriev et al., 2007) movement. Rab6 and its effector myosin II localize to Golgi bodies and promote fission of transport carriers that bears Rab6 (Miserey-Lenkei et al., 2010). The retrograde transport from late endosome is essential for recycling of Mannose-6-Phosphate receptors, which deliver lysosomal hydrolases from late endosomes/ lysosomes, back to Golgi. Rab9 via its effector TIP47 regulates retrograde transport of M6PR (Riederer et al., 1994; Carroll et al., 2001). However, a role in phagosome maturation for retrograde transport from the early endosome has not been demonstrated.

Rab18 has been shown to regulate the secretory pathway and the retrograde pathway from Golgi to ER (Dejgaard et al., 2008). During retrograde transport, Rab18 has been proposed to complement the function

of Rab6 and act as a tether prior to fusion of vesicles with ER (Dejgaard et al., 2008). While both Rab6 and Rab18 regulate secretory/Golgi to plasma membrane traffic, the defect in phagosome maturation is probably due to an unrelated function, as Rab1 and 8, regulators of the anterograde pathway from ER to Golgi and plasma membrane respectively (Plutner et al., 1991; Huber et al., 1993), do not affect phagosome maturation in our screen. Rab6 and Rab18 also regulate retrograde traffic, which is essential for varied physiological processes, such as insulin regulated trafficking, nutrient homeostasis in a cell, and Wnt signaling during development (Burd, 2011). Since the early endosome is the compartment from where cargo can be directed either for degradation or retrograde transport, whether there is any interaction between the two routes is an interesting question. Additional experiments validating our observation along with the specific targeting of retrograde traffic to examine the effect on phagosome maturation may shed more insight on this question.

Chapter 3

Rab14 regulates phagosomal maturation and is essential for the *Drosophila* immune response

Abstract

The removal or the functional disabling of hemocytes has been shown to increase fly susceptibility to *S.aureus,* but the underlying mechanism behind the observation remains poorly understood. As phagocytosis is a component of the cellular response, a phagosome maturation mutant *Rab14*^{null} was generated and its immune defect was characterized. We find that like Rab5 and Rab7, two well characterized regulators of the phagocytic pathway, the function of Rab14 in the hemocyte is essential for an effective host defense. Rab14 is required for efficient Rab7 recruitment onto the bacterial phagosome. Hence Rab14 is essential for progression of maturation and this possibly explains why Rab14 is a target for intracellular microbes. We also demonstrate that phagosome maturation is required to limit bacterial load and susceptibility to *S.aureus* in the animal. Along with that, phagosome maturation signals and regulates expression of specific AMP genes, possibly through the induction of the Toll ligand Spatzle. Thus the *Drosophila* immune response functions through a coordinated interaction between the cellular and humoral responses.

Introduction

A microbe-containing phagosome fuses with the early endosome, the late endosome and eventually with the lysosome, resulting in microbial clearance (Desjardins et al., 1994b). 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., 1994b) which in turn are essential for progression of phagosome maturation (Alvarez-Dominguez and Stahl, 1999; Rupper et al., 2001; Harrison et al., 2003). RabGTPases act as molecular switches — active in the GTP-bound form, while inactive in the GDP-bound form, the activity being regulated by guanine exchange factor (GEF) and GTPase activating protein (GAP). An active RabGTPase mediates its downstream effect through effectors (Grosshans et al., 2006). A wide array of RabGTPases is associated with phagosomes (Garin et al., 2001; Stuart et al., 2007), and each presumably contributes 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.

The *Drosophila* innate immune response is an ideal model system to gain such insights: the myriad of genetic tools and the ease with which *in vivo* studies can be done should help elucidate the role of genes regulating

phagosome maturation and also provide an understanding of its interaction with other components of the host immune response. The *Drosophila* cellular response is mediated by phagocytic hemocytes; their function is analogous to the mammalian macrophage. 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.

The humoral response leading to expression of antimicrobial peptides (AMPs) is the other component of the *Drosophila* innate immune response. During infection, recognition of Pathogen Associated Molecular Patterns (PAMPs) by Pattern Recognition Receptors (PRRs) leads to the activation of the Toll and Imd signaling pathways. These two pathways exhibit similarity to the mammalian TLR/IL-1R and Tumor Necrosis Factor-α (TNF-α) receptor signaling respectively (Ferrandon et al., 2007). The activation results in production of AMPs in the fat body, the liver equivalent in the fly, and later the AMPs are secreted into the hemolymph. Expression of the AMPs Drosomycin and Diptericin is typically used to indicate activation of the Toll and Imd pathways respectively. In Drosophila, Toll signaling is induced by fungi, gram-positive (G+) bacteria and Drosophila X Virus (Lemaitre et al., 1996; Michel et al., 2001; Zambon et al., 2005). The recognition of the pathogens or their virulence factors by the host, initiates a serine protease cascade which

culminates in activation of the Toll receptor ligand, Spatzle. The serine protease cascade leading to Spatzle activation has been extensively studied (Valanne et al., 2011). However, there is limited understanding of the regulation of Spatzle. Hemocytes play a key role in the expression of Spatzle in response to *Micrococcus luteus* infection (Shia et al., 2009) but the mechanism behind it is not known. Moreover, there is evidence both for (Brennan et al., 2007; Shia et al., 2009) and against (Brennan et al., 2007; Charroux and Royet, 2009; Defaye et al., 2009; Shia et al., 2009; Nehme et al., 2011) the possible contribution of hemocytes towards the humoral response.

Our study examines the role of phagosome maturation in the *Drosophila* immune response. During a pilot screen for mutants affecting phagosome maturation, Rab14 was identified as a candidate. Earlier proteomic studies detected Rab14 on latex bead-containing phagosomes, indicating a possible functional requirement during maturation (Garin et al., 2001). We also found that the *Rab14* transcript was upregulated on microarrays in response to fungal infection. Interestingly, Rab14 was required for *M.tuberculosis*-mediated phagosome maturation arrest in a human U937 macrophage-like cell line (Kyei et al., 2006). This indicated that Rab14 might be a target of intracellular bacteria and contribute to pathogenesis. Our preliminary observations indicated Rab14 is required by the host for an efficient cellular response. We hypothesized Rab14 regulates phagosome maturation at an essential stage and hence is targeted by intracellular

pathogens. The role that Rab14 plays during the cellular response to an extracellular pathogen is not known. To investigate Rab14's function, we generated a null mutation for *Rab14* and have characterized the immune defects in the mutant. By examining a mutant that has a defect in phagosome maturation, we also gain insight into the interactions between the hemocyte-mediated cellular response and the fat body-mediated humoral response.

Our study finds that phagosome maturation is essential to control bacterial load during *Staphylococcus aureus* infection in the fly. We have identified Rab14 as a positive regulator of phagosome maturation of *S.aureus* and *E.coli* and we demonstrate that Rab14 recruitment onto the phagosome follows Rab5 and precedes Rab7. A functional role for Rab14 in Rab7 recruitment was supported by Rab14's localization on early phagosomes and by the failure of efficient recruitment of Rab7 onto the phagosomes in the absence of Rab14. Our work is the first to show that Rab14 also localizes to Rab7-positive late endosomes and phagosomes, suggesting that Rab14 might act with Rab7, to regulate fusion of late phagosomes and lysosomes. The absence of Rab14 leads to a delay in phagolysosomal fusion and hence, a delay in maturation. Interestingly, Rab14 mutants also had defects in induction of specific AMPs in response to infection. We find that the Toll ligand Spatzle is indirectly regulated by phagosome maturation and thus provide evidence of the cross talk between cellular and humoral response.

Results

Rab14 and phagocytic machinery related RabGTPases are crucial for *S.aureus* defense

Rab14 was identified as a candidate in a pilot screen to identify genes regulating phagosome maturation; we asked what role it plays during an immune response. The *Drosophila* genome encodes three different *Rab14* isoforms; RA, RB and RC. The three isoforms show 100% identity to each other in the shared region. The isoforms RB and RC have an additional 24 or 18 amino acids at the N-terminus compared to RA. The isoforms all show 81% identity to human *Rab14* (Fig. 3-1A). RNA expression analyses detected only the RA and RB isoforms in adults (Fig.3-1B). The isoforms RA and RB were also expressed in the3rd instar larvae and in hemocytes (Fig. 3-1C). In order to see whether an infection leads to changes in Rab14 expression either in larvae or in hemocytes, larvae were infected with *E.coli*. Real-time PCR data indicates no difference in expression of Rab14 RA following infection in larvae or hemocytes (Fig. 3-1D). Rab14 RB also showed no difference in larvae upon infection. However, since Rab14 RB transcript is in low abundance, its expression data is sensitive to small differences in the hemocyte samples. Hence for hemocytes, no conclusion with respect to Rab14 RB induction could be obtained.

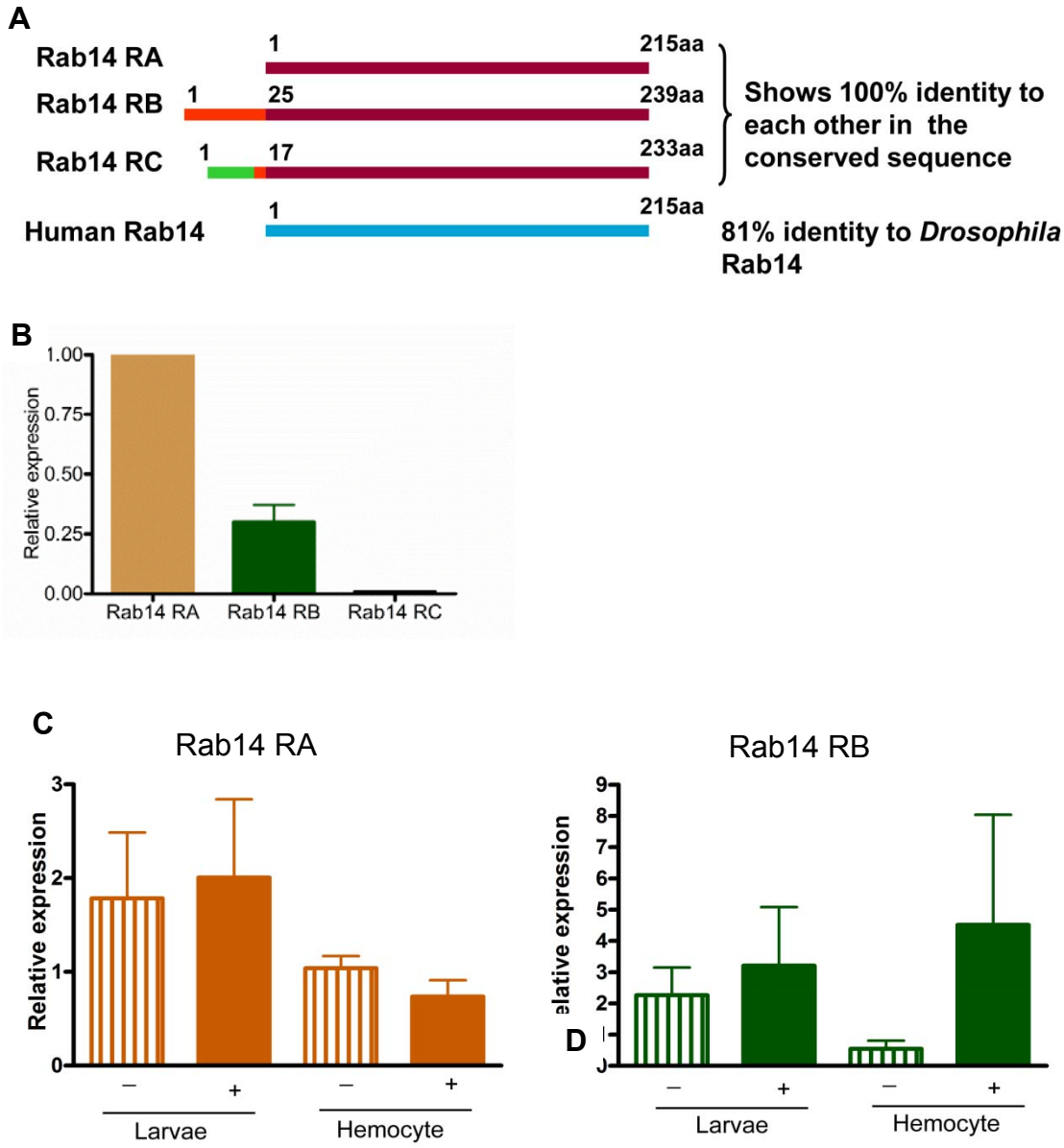

Figure 3-1. Expression of *Rab14* isoforms in *Drosophila* (A) A comparison of *Rab14* isoforms expression in adults. (B) Isoform specific primers of comaparble efficiency were used to analyze expression of *Rab14* isoforms RA, RB and RC using Real time PCR. (C) Expression of Rab14 RA or (D) RB were examined in larvae or in hemocytes 6h after injection with, ─ PBS (wounding control) or + *E.coli*. Error bars indicate standard deviation (SD), n=3. The experiments were repeated at least 3 times.

Isoform-specific functions during phagosome maturation have been observed for other RabGTPases (Alvarez-Dominguez and Stahl, 1999), we were interested in examining functional similarities or differences between the Rab14 isoforms. A *Rab14*^{null} mutant is a prerequisite for dissecting out functions of the two isoforms. Neither of the two existing *Rab14* transposon lines completely eliminated *Rab14* expression (Fig.3-2A, B). The two transposon lines, EY04032 and BG01134 conveniently flanked the *Rab14* gene, allowing the generation of a *Rab14* mutant using transposase-induced recombination (Fig. 3-2C). Real-time PCR demonstrates *Rab14* mutant lacks expression of both the isoform RA and RB (Fig. 3-2A, B). Sequencing of the genomic DNA in the mutant indicated two excision sites: one within the first transposon EY04032 and the other at the end of the second transposon BG01134.

The *Rab14* mutants showed no apparent morphological defects and were fertile. Additionally expression of the AMPs, Drosomycin and Diptericin in response to infection was not affected indicating there was no global defect in the Toll or Imd pathways (Fig.3-3A, B).

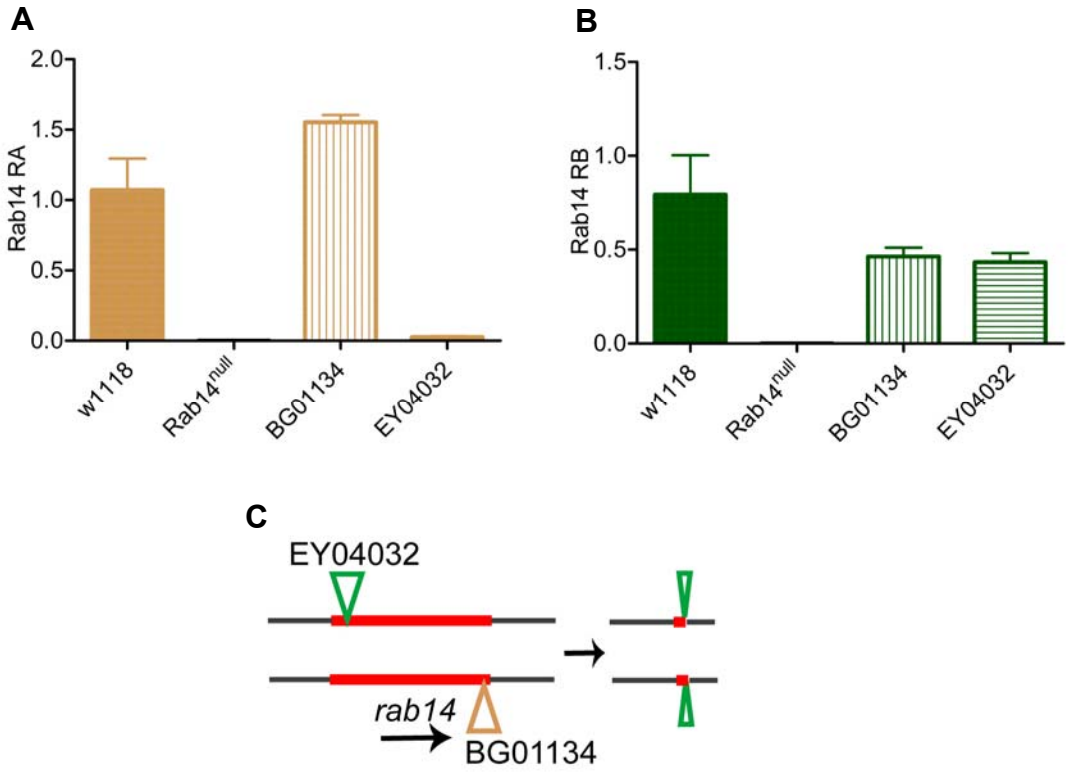

Figure 3-2. Generation of a *Rab14* mutant (A) A comparison of *Rab14* isoforms relative expression in the transposon insertion lines used for generation of the *Rab14* mutant (EY04032 and BG01134) and in the *Rab14* mutant using Real-time PCR. (E) A simplified representation of transposon location with respect to *Rab14* gene. Transposase-induced recombination between the transposons EY04032 and BG01134 in males led to the generation of a $Rab14^{null}$ mutant. Error bars indicate standard deviation (SD), n=3. The experiments were repeated at least 3 times.

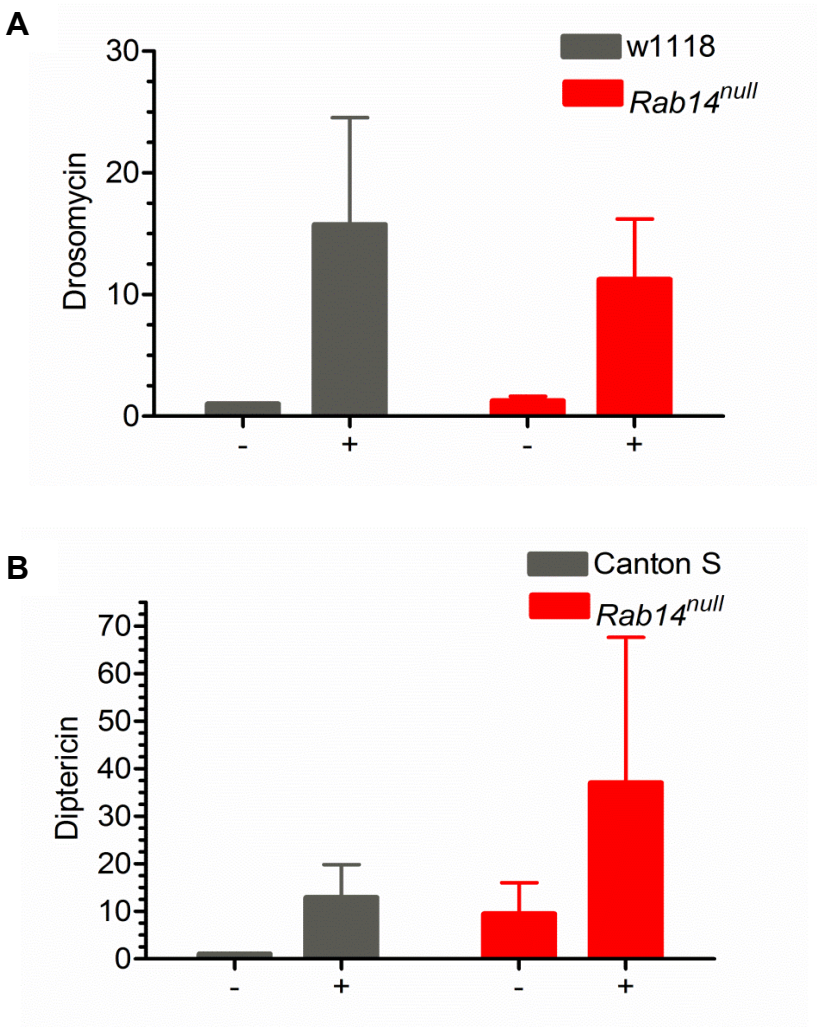

Figure 3-3. Induction of Drosomycin and Diptericin is not affected in *Rab14* mutants Wild type and *Rab14* mutants were injected with (+)*S.aureus* or (+) *E.coli*. Induction of (A) Drosomycin was examined 24h after *S.aureus* infection (B) Diptericin was examined 6h after *E.coli* infection. PBS injection (-) was used as wounding control. Error bars indicate SEM, n=3. The experiments were repeated 3 times.

To understand the consequences of Rab14 loss on the ability of the fly to withstand infections, survival studies were carried out. *Rab14* mutants were not susceptible to *E.coli* infection (Fig.3-4A). This is not surprising, as *E.coli*-induced Diptericin expression is unaffected in the *Rab14* mutant (Fig.3-3B). An increased susceptibility to non-pathogenic *E.coli* would require a more complete absence of both the cellular and humoral responses (Elrod-Erickson et al., 2000). Next, we looked at the survival of *Rab14* mutants over a time period following infection with *S.aureus*. *Rab14* mutants were significantly more susceptible to *S.aureus* (Fig.3-4B). For the rescue and cell biology studies, a transgenic line expressing Rab14 RB-mRFP was generated. For isoform RA, a Rab14 RA-YFP line was obtained from the Bloomington stock center (Zhang et al., 2007). Expression of either *Rab14* isoform RA or RB specifically in the hemocytes rescues the susceptibility of the $Rab14^{null}$ mutant (Fig.3-4B). Hence both isoforms are capable of rescuing *Rab14* loss suggesting they have similar functions. Importantly, our rescue data suggests that the susceptibility in *Rab14* mutants is due to Rab14 function in hemocytes.

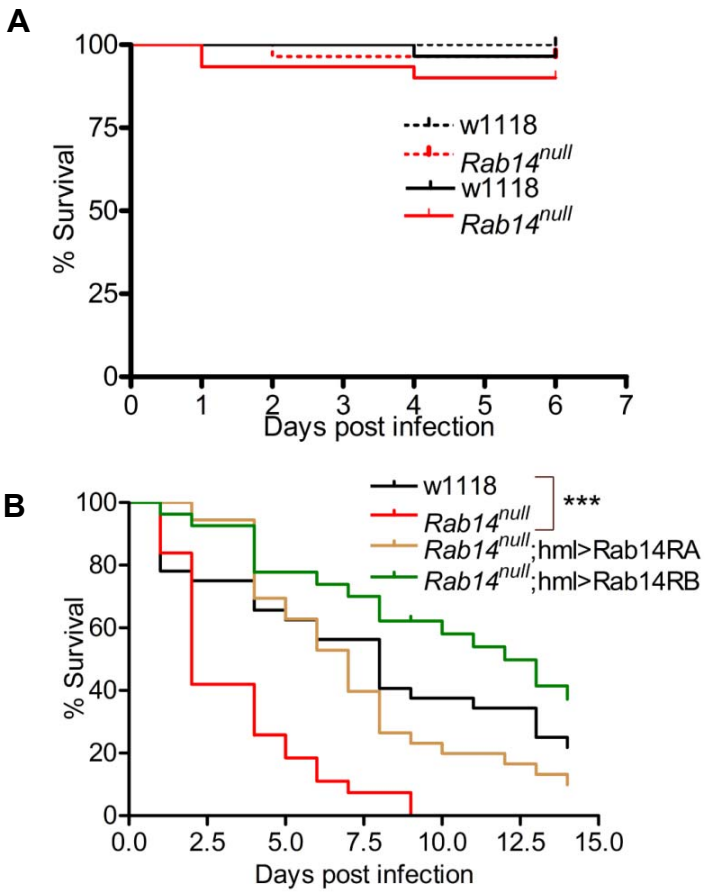

Figure 3-4. *Rab14* mutants are susceptible to *S.aureus* infection (A) Survival curves following *E.coli* infection in wildtype and *Rab14* mutants. A concentrated log phase culture of *E.coli* resuspended in PBS was used for infections. Similarly concentrated, heat inactivated *E.coli* was used as control. Dashed and solid lines in survival curve represent wounding control and infection respectively. (B) Survival curves following *S.aureus* infection in wildtype and *Rab14* mutants. The rescue studies were carried out by expressing *Rab14* isoform RA or RB in the background of the *Rab14* mutant using hmlGAL4. A log phase culture of *S.aureus* was used for infections. *** indicates $p < 0.001$. The experiments were repeated at least 3 times.

The activity of a Rab GTPase is mainly attributed to the GTP-bound form and not the GDP-bound form. Since a *Rab14* mutant lacks both forms, in order to ascertain if the increased susceptibility of *Rab14* mutants to *S.aureus* is due to lack of an active Rab14, we utilized a Rab14 Dominant-Negative (DN) mutant (S49N) (Zhang et al., 2007). Hemocyte-specific expression of a Rab14DN 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.3-5A). Furthermore, ubiquitously downregulating Rab14 activity rather than doing so only in the hemocytes did not cause increased susceptibility. This further corroborates that Rab14's role in the hemocyte alone plays an important role during the *S.aureus* immune response.

Rab5 and Rab7 are known regulators of phagosome maturation. Hemocyte-specific down-regulation of either *Rab5* or *Rab7* in the hemocytes also increased susceptibility to pathogenic *S.aureus* suggesting that phagosome maturation plays an essential role in limiting infection (Fig.3-5B). The increased susceptibility to *S.aureus* upon Rab5 or Rab7 downregulation is similar to that observed in Rab14 mutants. This observation was consistent with our hypothesis of Rab14 acting as a positive regulator of phagosome maturation like Rab5 and Rab7. Hence we focused on examining the process of phagocytosis and maturation in the *Rab14* mutants.

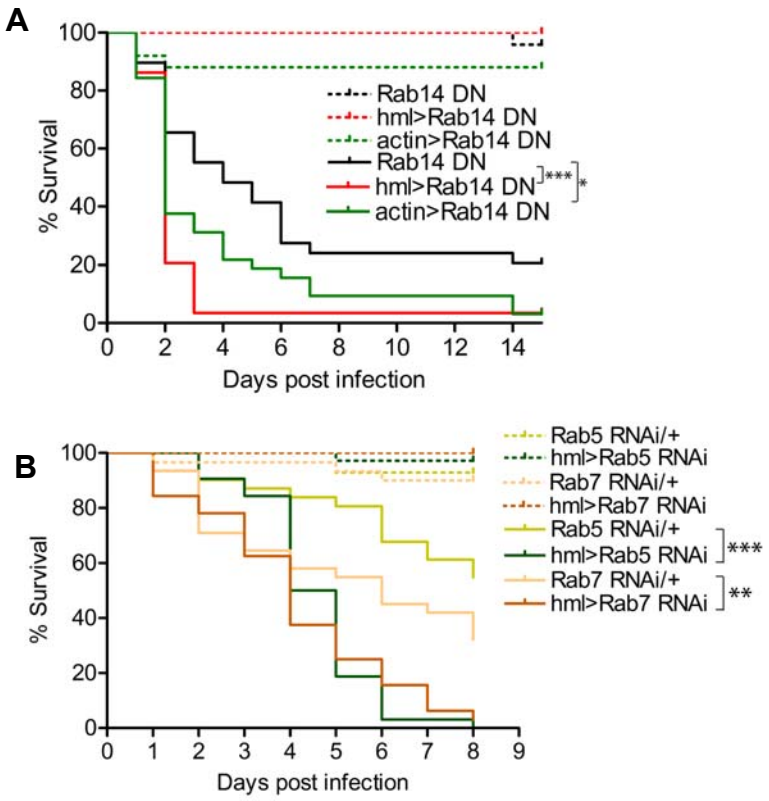

Figure 3-5. Endocytic machinery is essential for the immune response against *S.aureus* (A) Survival curves following *S.aureus* infection upon expression of Rab14DN ubiquitously (actinGAL4) or only in hemocytes (hmlGAL4). The Rab14 line outcrossed to w1118, the parental line of hmlGAL4 was used as a control. (B) Survival curves following *S.aureus* infection upon expression of Rab5 RNAi or Rab7 RNAi in hemocytes (hmlGAL4). The Rab5 RNAi line outcrossed to w1118, the parental line of hmlGAL4 was used as a control. The dashed and solid lines in the survival curves represent wounding controls and Infection respectively. At least 30 flies were injected with log phase culture of *S.aureus* and survival was assessed every 24 h. The experiment was repeated 3 times. * $p \leq 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Rab14 is required for phagosome maturation of *S.aureus* and *E.coli*

Phagocytosis results in clearance of microbes (Haine et al., 2008) which otherwise would multiply, resulting in lethality to the host. The lethality associated with microbial infection is often attributed to high bacterial load however a decrease in ability to tolerate the damage caused by the microbe can also be the reason behind lethal infection(Schneider and Ayres, 2008). To determine whether the increased death of *Rab14* mutants following *S.aureus* infection was due to defective resistance or decreased tolerance 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 this could be rescued with hemocyte-specific expression of either isoform (Fig.3-6). Hence the increased susceptibility of the *Rab14* mutant is due to the inability of the *Rab14* deficient hemocytes to limit bacterial growth.

To assess what role Rab14 might be playing in the hemocytes, we examined phagocytosis and phagosome maturation of fluorescence-conjugated *S.aureus* or *E.coli* in adult *Rab14* mutants. In *Drosophila*, the hemocytes along the dorsal vessel can be visualized through the cuticle and provide a means to follow phagocytosis and maturation *in vivo* (Elrod-Erickson et al., 2000). The uptake of fluorescein-conjugated *S.aureus* (Fig.3-7A, C) and *E.coli* (Fig.3-7B, D) was comparable in wildtype and mutant, suggesting Rab14 is not required for initial phagocytic uptake.

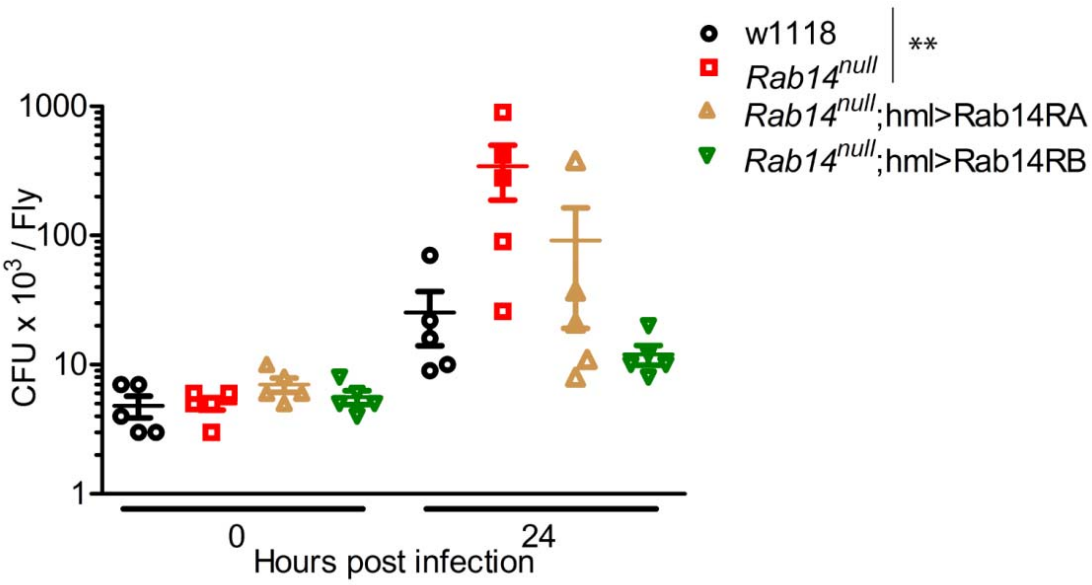

Figure 3-6. The susceptibility of *Rab14* mutants is associated with high bacterial loads Wildtype, *Rab14* mutants, and hemocyte-specific rescue lines expressing *Rab14* isoforms RA or RB in the *Rab14* mutant background were injected with *S.aureus.* Flies were collected immediately or 24h after infection. Surface-sterilized flies were homogenized, plated and counted for colony forming units. Each dot represents data from 3 pooled flies. ** $p < 0.01$

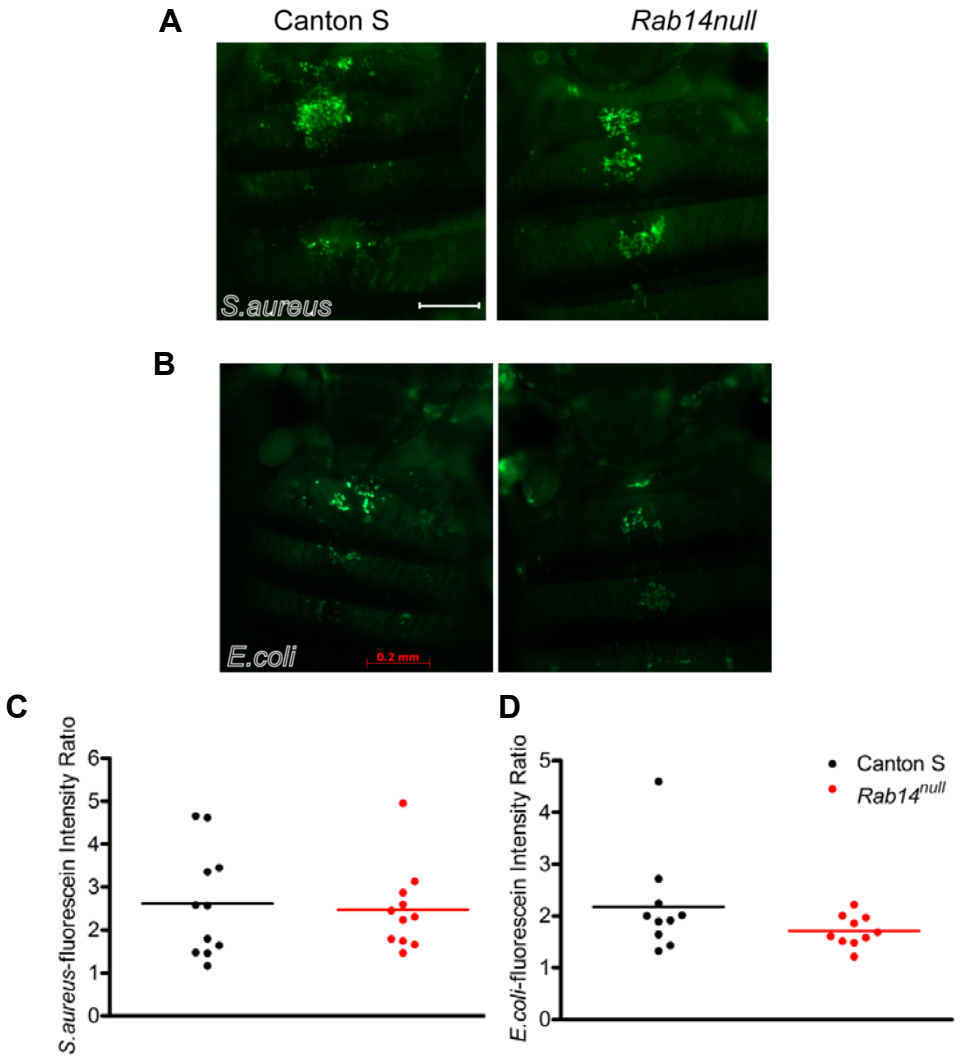

Figure 3-7. Rab14 is not required for phagocytic uptake A representative image for comparison of phagocytosis 30min after injection of fluorescein-conjugated (A) *S.aureus* or (B) *E.coli* between wildtype and *Rab14* mutants. Images were obtained immediately following Trypan blue quenching of extracellular fluorescence. Quantification of (C) *S.aureus* or (D) *E.coli* associated fluorescence intensity in the dorsal vessel plotted on a scatter plot. Each dot represents the intensity associated with one fly. Scale bar 0.2mm. The experiment was repeated 3 times.

This result confirms *in vivo* the work of Kim and group who carried out a phagocytosis screen to identify RabGTPases involved in the uptake of *E.coli* in Drosophila SL2 cells (Shim et al., 2010). They found *Rab14* down-regulation does not affect the uptake of *E.coli*. Next, we examined phagosome maturation in the *Rab14* mutant using pHrodo-conjugated microbial particles (Cuttell et al., 2008). The pHrodo dye is sensitive to pH: it is non-fluorescent at neutral pH and its fluorescence intensity increases with an increase in acidity of the phagosome. The progression of phagosome maturation involves fusion of a microbe-containing phagosome with increasingly acidic compartments. Thus following uptake by the hemocytes, pHrodo-conjugated microbes will show an increase in fluorescence intensity as maturation progresses.

Wildtype and *Rab14* mutant both showed significant increase in intensity with increase in timepoints (30, 60 and 90min). However *Rab14* mutants showed lower intensity as compared to wild type at each timepoint. This suggests *Rab14* mutants demonstrate slower kinetics for maturation of pHrodo-*S.aureus* until 90 min post-injection (Fig.3-8A, B). The defect in maturation of *S.aureus* could be rescued with hemocyte-specific expression of *Rab14 RA*, suggesting that the defect in maturation was specifically due to loss of Rab14 function in the hemocytes (Fig.3-9A, B). Rab14 also plays an essential role in maturation of *E.coli* phagosomes (Fig.3-10A, B). Hence the phagocytic machinery seems to be conserved for these different classes of bacteria.

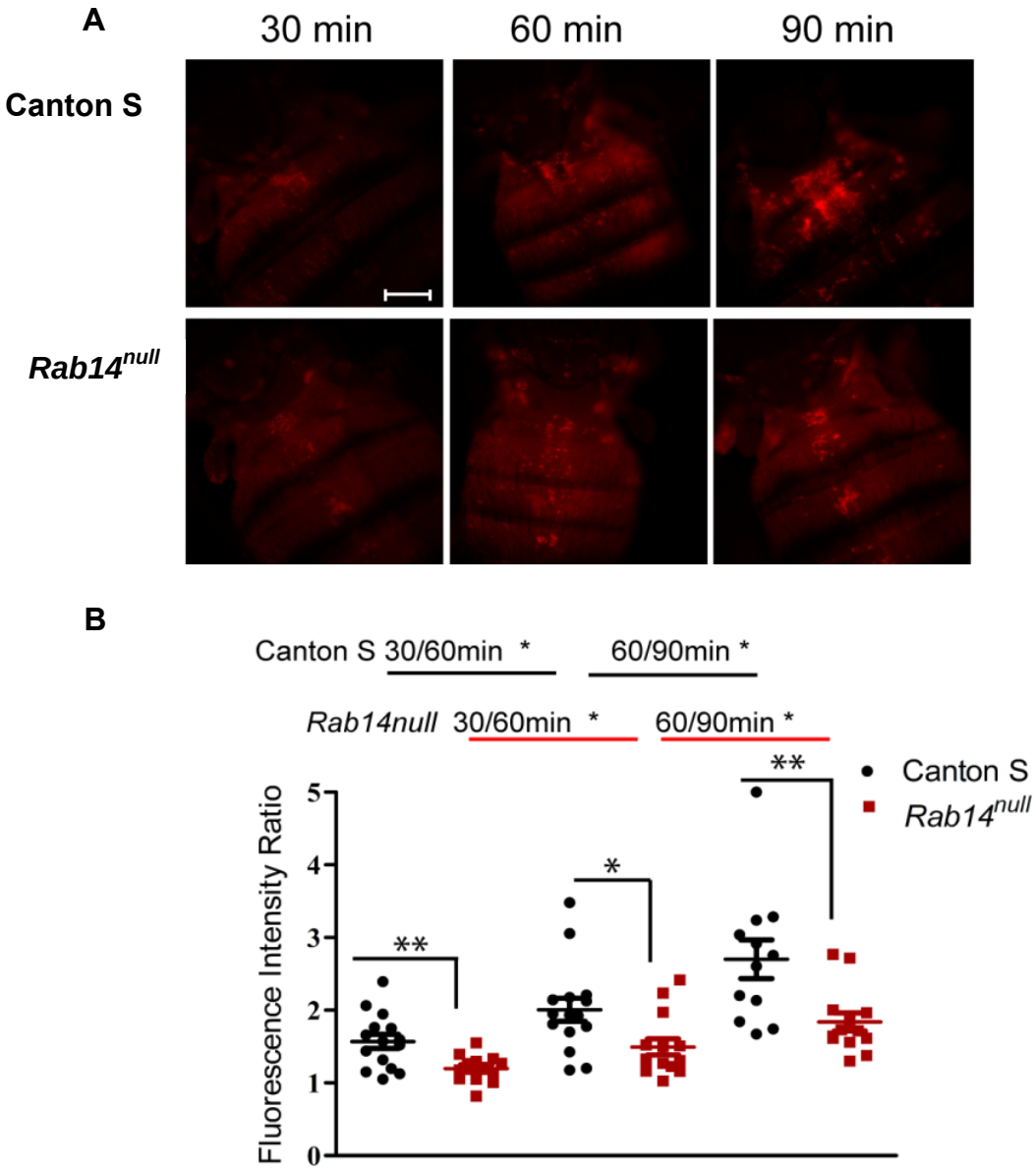

Figure 3-8. *Rab14* mutants display slower kinetics of phagosome maturation A representative (A) image and (B) quantification of phagosome maturation. Flies were injected with pHrodo-conjugated *S.aureus* and images were taken at 30, 60 or 90 min post-injection. Scale bar 0.2mm. * $p \leq 0.05$, ** $p < 0.01$

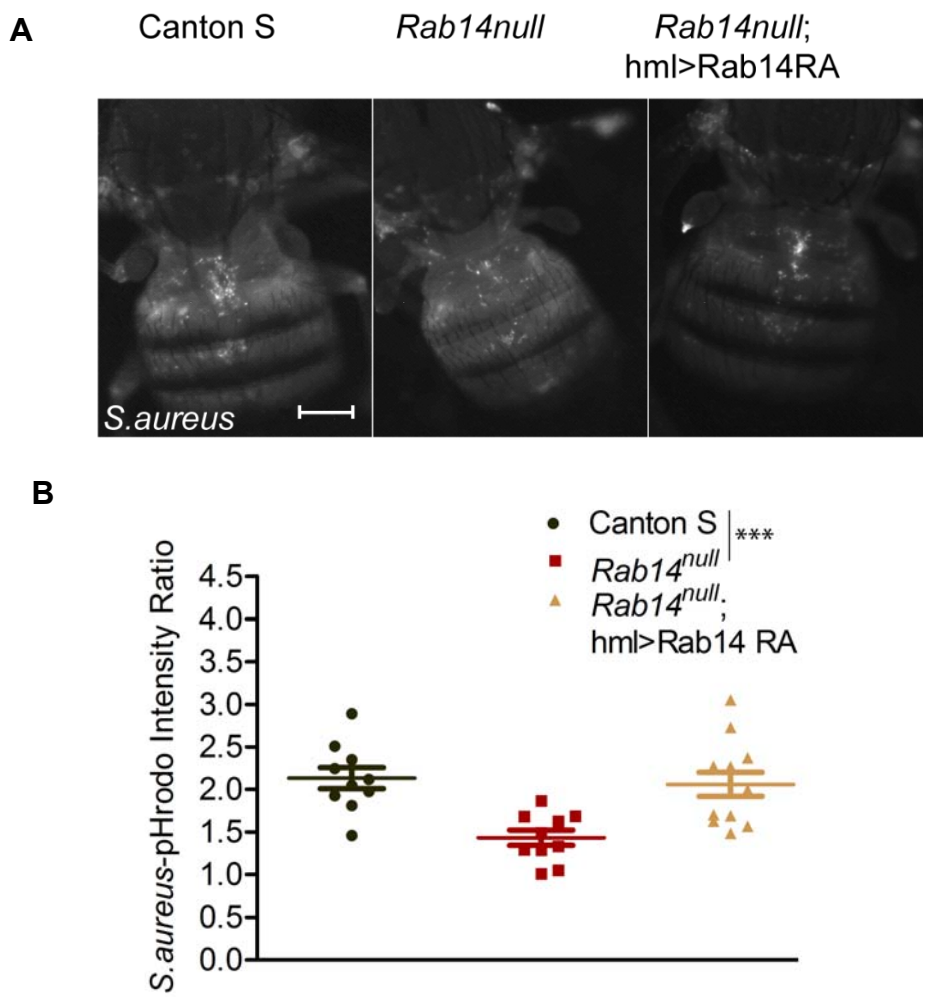

Figure 3-9. Rab14 is essential for phagosome maturation of *S.aureus*

Wildtype, *Rab14* mutants and a rescue line expressing *Rab14* RA in hemocytes were injected with pHrodo-conjugated *S.aureus* and images were taken after 60min. (A) A representative image and (B) quantified fluorescence intensities are shown. The experiments were repeated 3 times. Scale bar 0.2mm. *** $p<0.001$

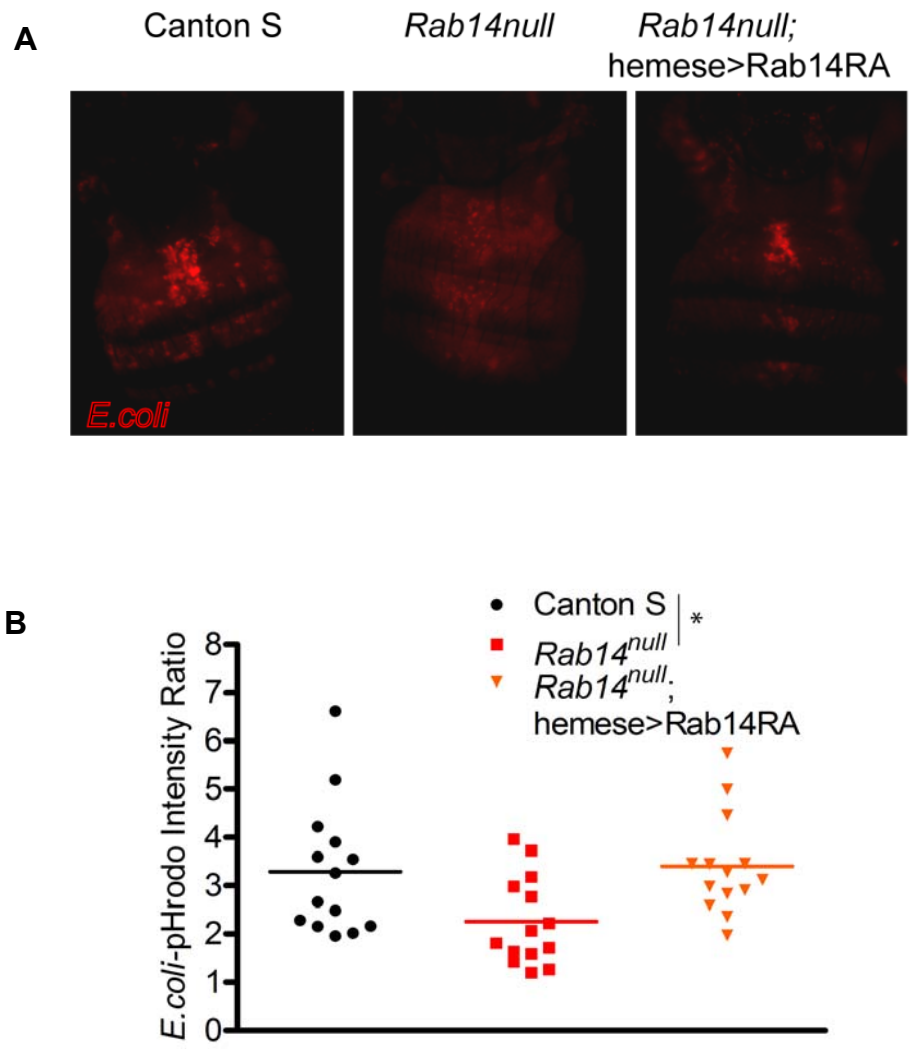

Figure 3-10. Rab14 is essential for phagosome maturation of *E.coli*.

Wildtype, *Rab14* mutants and a rescue line expressing *Rab14* RA in hemocytes were injected with pHrodo-conjugated *E.coli* and images were taken after 60min. (A) A representative image and (B) quantifed fluorescence intensities are shown. The experiments were repeated 3 times. Scale bar 0.2mm. * $p < 0.05$

Rab14 shows partial co-localization with both Rab5 and Rab7

Cellular localization provides a glimpse to the possible function of a protein. If Rab14 is a regulator of phagosome maturation, it should be associated with phagocytic and/or endocytic machinery. To determine the identity of the Rab14 compartment, we examined the co-localization of Rab14 RB with Rab5 and Rab7. In mammalian cells, Rab5-positive early endocytic structures are smaller and are localized more towards the periphery of the cell while Rab7-positive late endosomal structures are larger and are more perinuclear (Rink et al., 2005).

The hemocyte population of *Drosophila* adults are mostly sessile, on the other hand larvae are a good source of circulating hemocytes. Hence for *ex vivo* hemocyte study we used larval hemocytes. We could localize mRFP-tagged Rab14 to both types of endocytic structures. As Drosophila hemocytes are small cells (10um), the differential distribution of the endosomes within the cells is not as obvious. In order to stimulate the phagocytic machinery, we injected larvae with *E.coli* and harvested hemocytes after 15 or 45 min. The mRFP-tagged Rab14 RB showed partial co-localization with GFP-tagged Rab5 in early endsomal/phagosomal compartments of larval hemocytes (Fig.3-11). A second endosomal marker, FYVE-GFP also co-localized with Rab14 RB, further confirming its early endosomal localization (Fig.3-12A). The same figure also shows a larger endosomal-like compartment with Rab14 RB localization but lacking FYVE-GFP, suggesting that it might be a late endosome. Rab14 localization on Rab7-positive compartments in

hemocytes validated Rab14 presence on Rab7-containing late endosomal/phagosomal compartments in hemocytes (Fig.3-11). Additional smaller Rab14 postive but Rab7 negative structures might be early endosomes. However, unlike Rab5 and Rab7, Spinster-GFP (Sweeney and Davis, 2002), a lysosomal marker, rarely showed co-localization with Rab14, suggesting that Rab14 is not found on lysosomes (Fig.3-11).

Confocal microscopy of the non-phagocytic tissue fat body indicated GFP-tagged Rab5 and mRFP-Rab14 co-localized at the periphery of the cell (Fig. 3-12B). Imaging at a nuclear focal plane indicated Rab14 also co-localized with Rab7-GFP (Fig. 3-12B). Hence, like its mammalian homolog, *Drosophila* Rab14 is also found on the 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 transient association of RabGTPases with membranes and hence its presence on late endosomes might have been missed in the earlier study. To determine if there might be differences between the Rab14 isoforms RA and RB, we looked at the localization of fluorophore-tagged proteins in the larval hemocytes (Fig.3-13). Both the isoforms showed similar localization patterns consistent with overlapping functions as seen in rescue of survival and bacterial load studies.

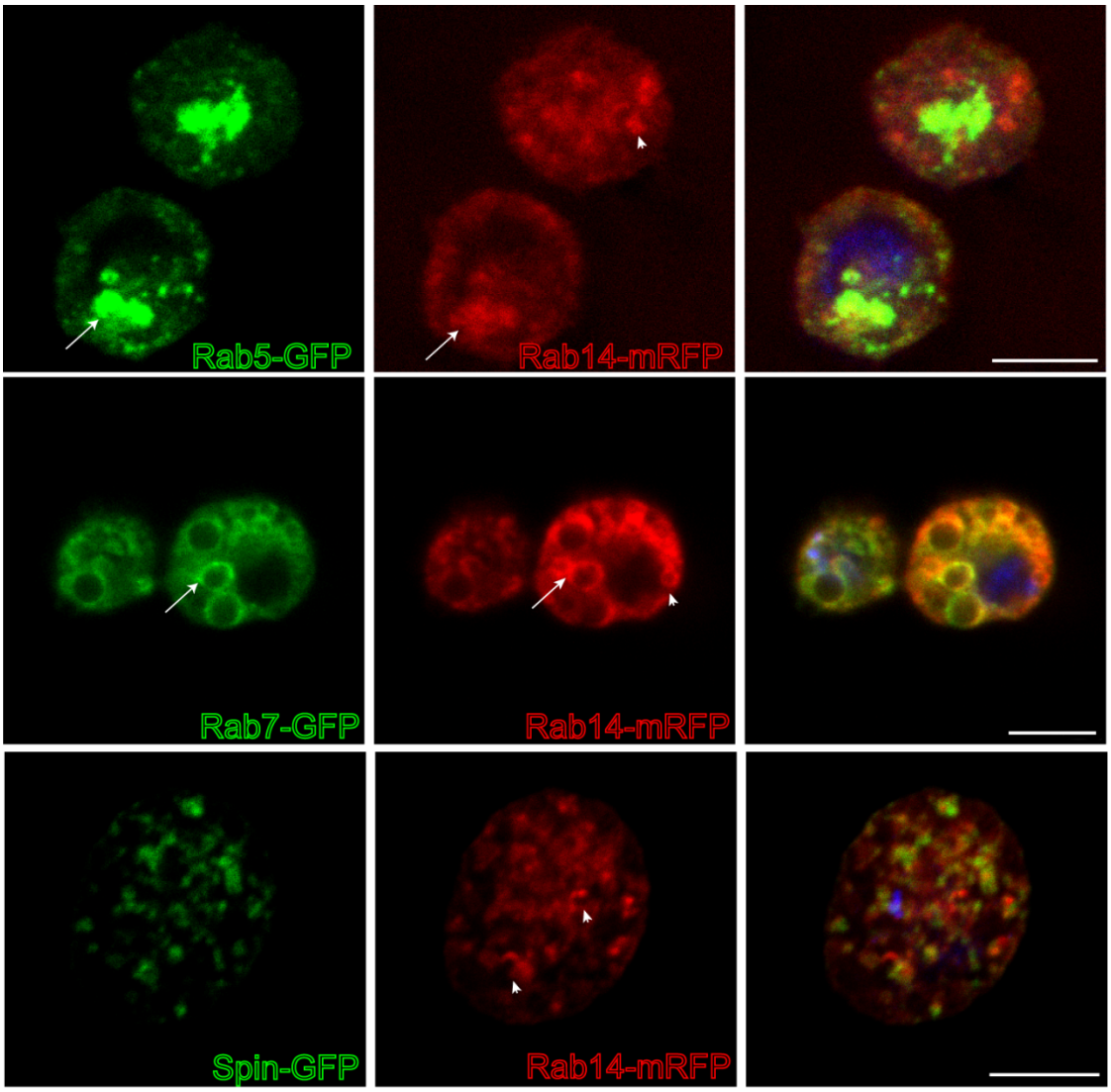

Figure 3-11. Rab14 shows partial co-localization with early and late endosomal markers in hemocytes Larvae expressing Rab14 RB-mRFP and (top panel) Rab5 GFP or (middle panel) Rab7 or (bottom panel) Spinster GFP in hemocytes were injected with *E.coli.* After 15min (top panel) or 45min (middle and bottom panel) larvae were bled and hemocytes were fixed and imaged. Arrows and arrowheads indicate areas of colocalization or no colocalization between Rab14 RB and the markers respectively. Bar 5 μm

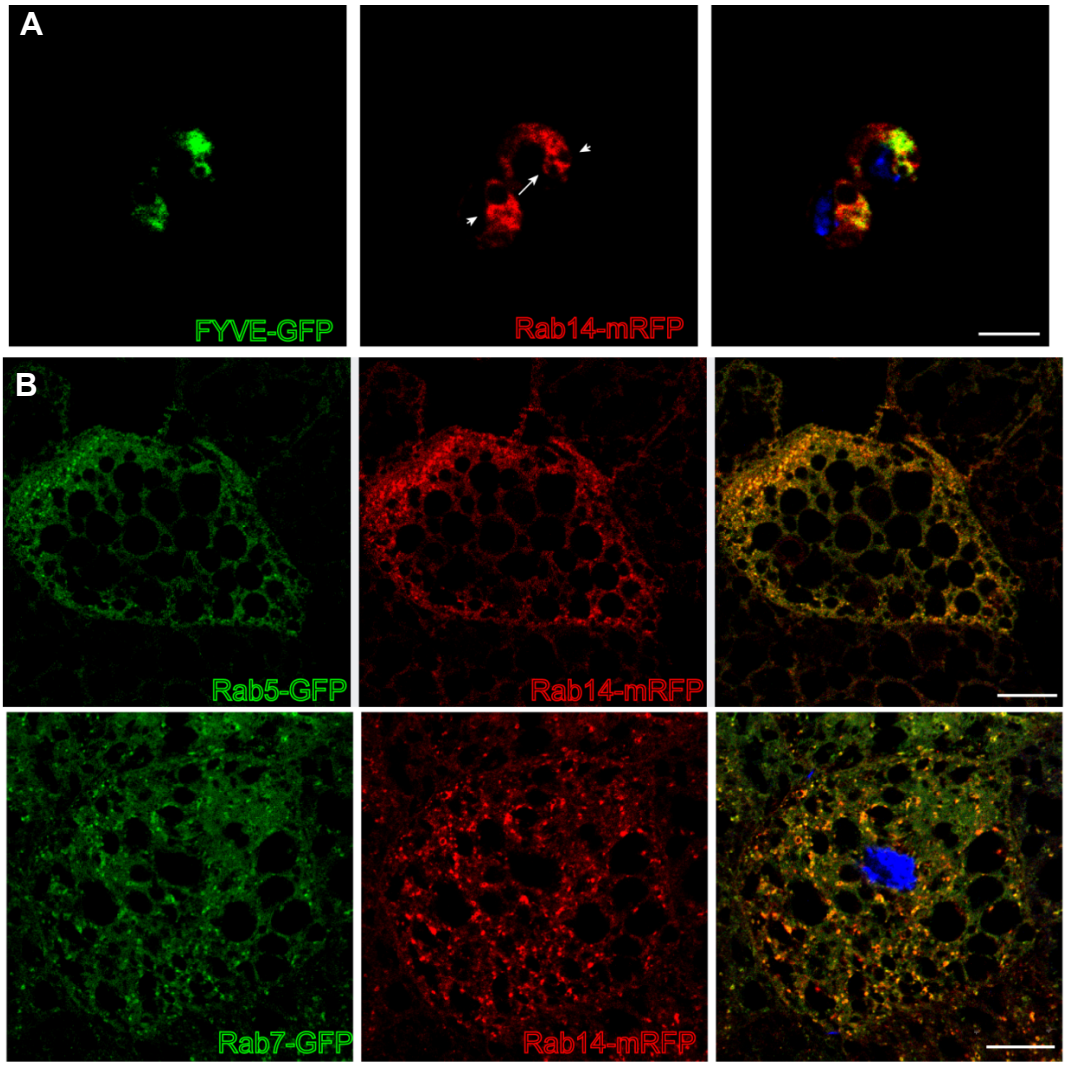

Figure 3-12. Rab14 shows partial co-localization with early and late endosomal markers (A) Larvae expressing Rab14 RB-mRFP and FYVE-GFP in hemocytes were injected with *E.coli* and hemocytes were bled out after 15min and fixed. Scale bar 5 μm (B) Larval fat body expressing Rab14 RB-mRFP with Rab5-GFP (top panel) and Rab7-GFP (bottom panel) were dissected out and fixed. Scale bar 20 μm. Arrows and arrowheads indicate areas of colocalization or no colocalization between Rab14 RB and the markers respectively.

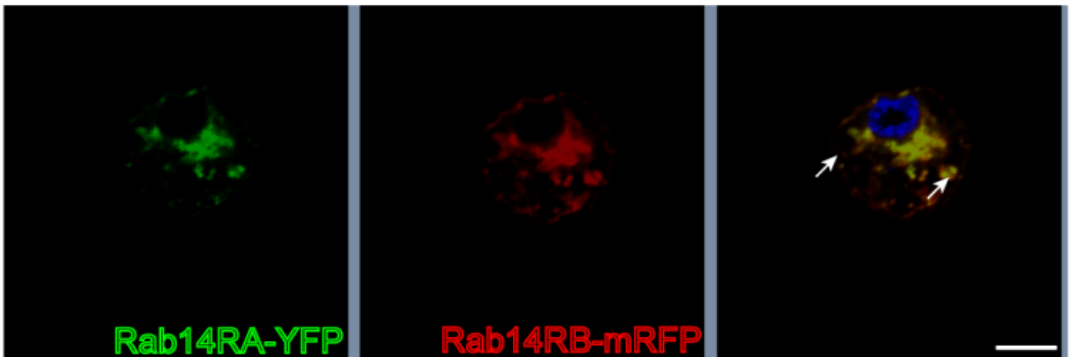

Figure 3-13. Colocalization of Rab14 isoforms Hemocytes from larvae expressing tagged isoforms Rab14 RA-YFP and Rab14 RB-mRFP were bled out and fixed. Scale bar 5 μm

Decreased recruitment of late endosomal and lysosomal markers in *Rab14* mutants

The localization of Rab14 on the endosomes indicated it might be playing a role in maturation by regulating vesicular trafficking. The newly formed phagosome interacts with the early endosome, late endosome and then lysosome which leads to acidification of the phagosome (Vieira et al., 2002). The recruitment of RabGTPases is essential for the sequential maturation. To understand the role of Rab14, we asked at what stage is phagosome maturation affected in the mutant. We examined the recruitment of Rab5, Rab7 and the lysosomal marker Spinster (Sweeney and Davis, 2002) onto the *S.aureus* phagosome in hemocytes of wildtype and *Rab14* mutants. After a 20 min pulse of *S.aureus*, phagosomes from both wildtype and *Rab14* mutants recruited Rab5 in a comparable manner (Fig.3-14A, B). This suggested that phagosome maturation is normal at the early endosome stage. Next, we looked at Rab7-positive phagosomes after a 10 min chase following the 20 min pulse. When compared to wild type (40.67±1.45%), *Rab14* mutants showed significantly less Rab7 recruitment (26.0±2.3%) (Fig. 3-15A, B). Additionally, whereas relatively high numbers of bacteria showed co-localization in the wild type with the lysosomal marker Spinster (65.0±5.4%), significantly less (35.0 ±7.1%) Spinster-positive phagosomes were observed in the *Rab14* mutant (Fig. 3-16A, B). The data indicate that Rab14 plays a role in recruitment of Rab7. The recruitment of Rab7 onto phagosomes is essential for subsequent fusion with late endosomes. This

suggests that the decreased maturation in the mutant is due to delayed fusion with late endosomes.

Considering the important role Rab14 plays during maturation, we examined the recruitment of Rab14 RB-mRFP to *S.aureus* phagosomes (Fig.3-17A). 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 Rab14 presence was reduced on phagosomes following a 20 min chase (Fig.3-17B). Rab14 RA-YFP showed similar recruitment to phagosomes of *E.coli* (Fig.3-17C). Thus the timeline of recruitment of Rab14 onto the phagosomes matches with that of both Rab5 and Rab7. This is consistent with Rab14 being present on both early and late endosomes (Fig.3-11). The presence of Rab14 on the early phagosome is consistent with its functional requirement for Rab7 recruitment. Rab14's presence on both the late endosome (Fig.3-11) and late phagosome (Fig.3-17A, B) and the failure of Rab14 mutant phagosomes to mature to phagolysosomes, indicate that Rab14, like Rab7 might regulate the fusion of phagosomes with late endosomes.

Endosomal maturation in the fat body is affected in *Rab14* mutants

Rab7 recruitment is essential for both phagosomal and endosomal maturation (Feng et al., 1995; Harrison et al., 2003). We therefore asked whether Rab14 also plays a role in both the phagocytic and endocytic pathways.

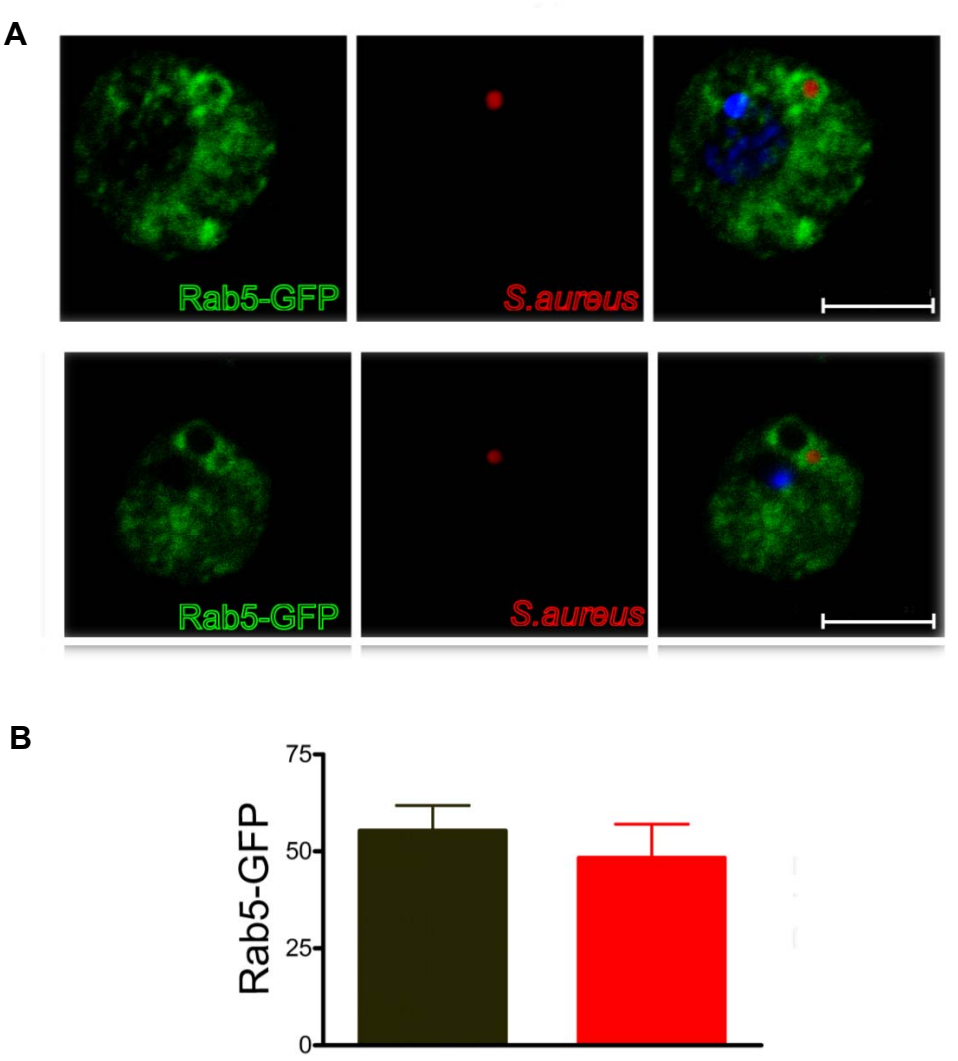

Figure 3-14. Rab5 recruitment on the phagosome is not affected in *Rab14* mutants (A) Larval hemocytes from wildtype and the *Rab14* mutant were bled out 20min after infection with *S.aureus* conjugated to AF-594 (red). They were evaluated for phagosomal recruitment of the early endosomal marker Rab5 tagged with GFP. (B) Percentage of phagosomes showing recruitment of Rab5 GFP is plotted for wildtype and $Rab14^{null}$. Error bars indicate standard error of the mean (SEM), n = 3. Scale bar, 5μm * $p \leq 0.05$.

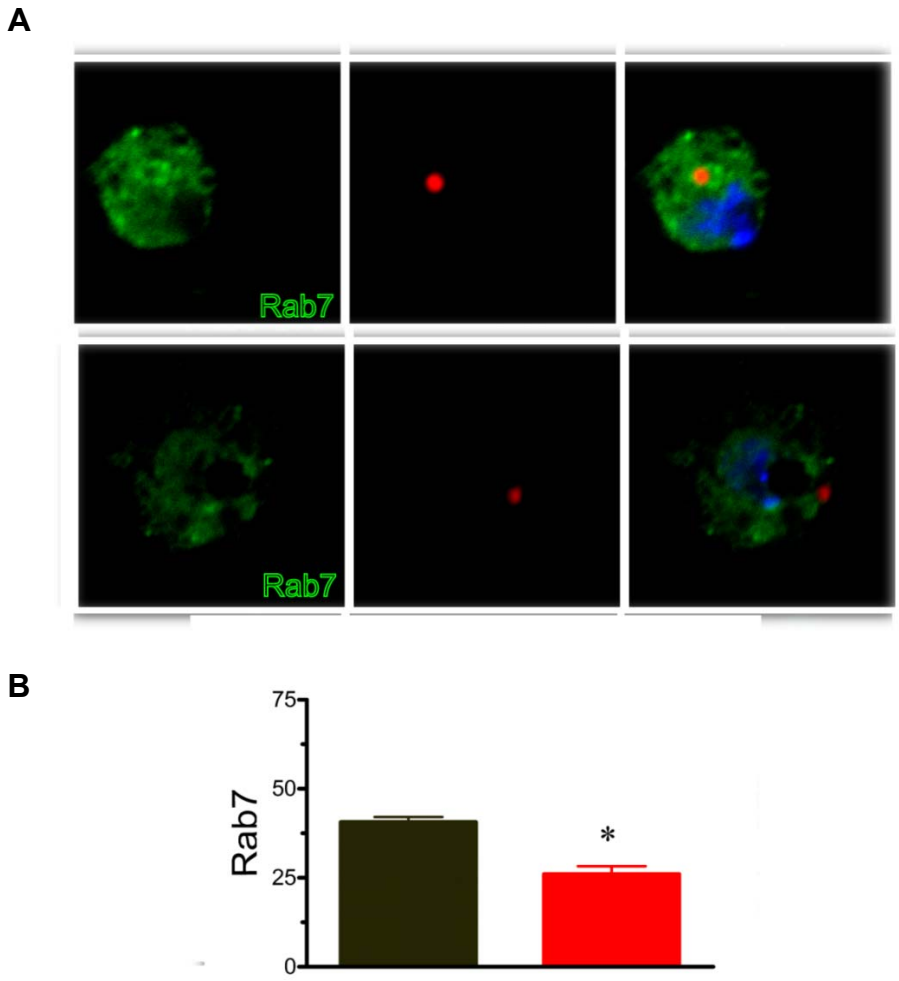

Figure 3-15. Decreased recruitment of the Rab7 on to the phagosome of *Rab14* mutants (A) Larval hemocytes from wildtype and the *Rab14* mutant were bled out after a 10 min chase following 20 min pulse with *S.aureus* conjugated to AF-594 (red). They were evaluated for phagosomal recruitment of the late endosomal marker Rab7 using a Rab7 antibody. (B) Percentage of phagosomes showing recruitment of Rab7 is plotted for wildtype and $Rab14^{null}$. Error bars indicate standard error of the mean (SEM), n = 3. Scale bar, 5μm * $p \leq 0.05$.

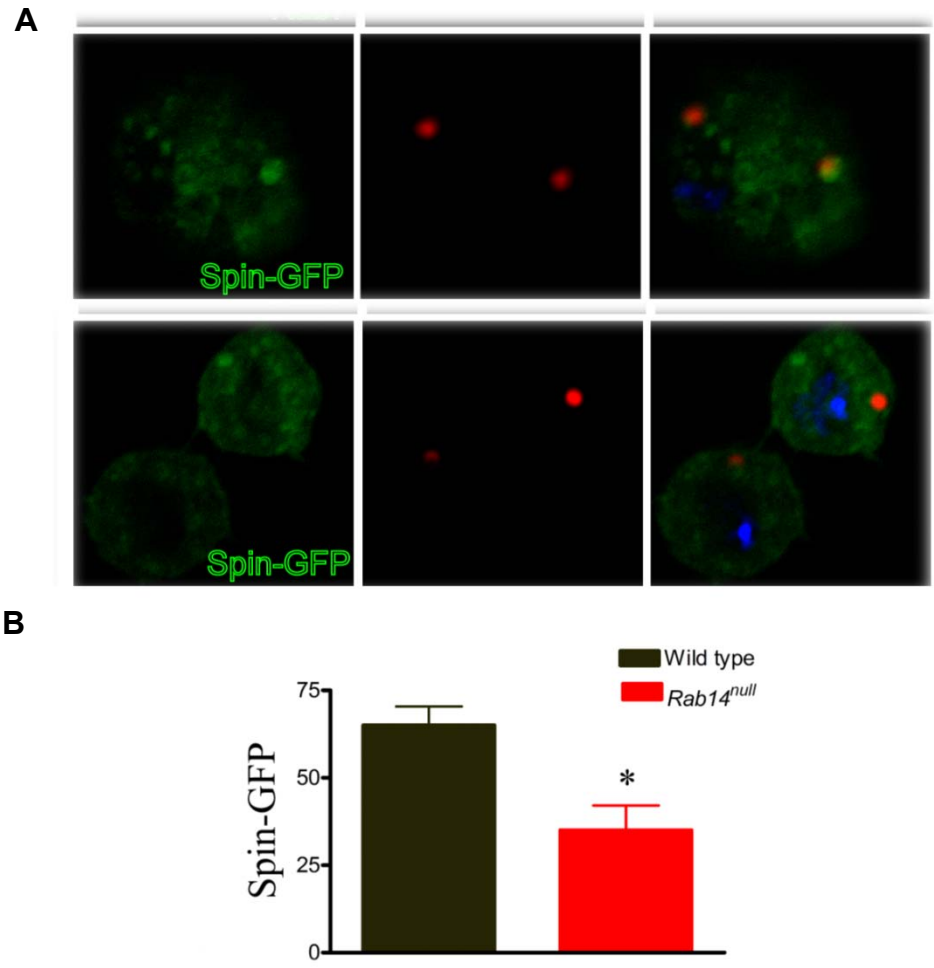

Figure 3-16. Decreased recruitment of the lysosomal marker to the phagosomes of *Rab14* mutants (A) Larval hemocytes from wildtype and the *Rab14* mutant were bled out after 20 min chase following 20 min pulse with *S.aureus* conjugated to AF-594 (red). They were evaluated for phagosomal recruitment of the lysosomal marker Spinster tagged with GFP. (B) Percentage of phagosomes showing recruitment of the respective markers is plotted for wildtype and $Rab14^{null}$. Error bars indicate standard error of the mean (SEM), n = 3. Scale bar, 5μm * $p \leq 0.05$.

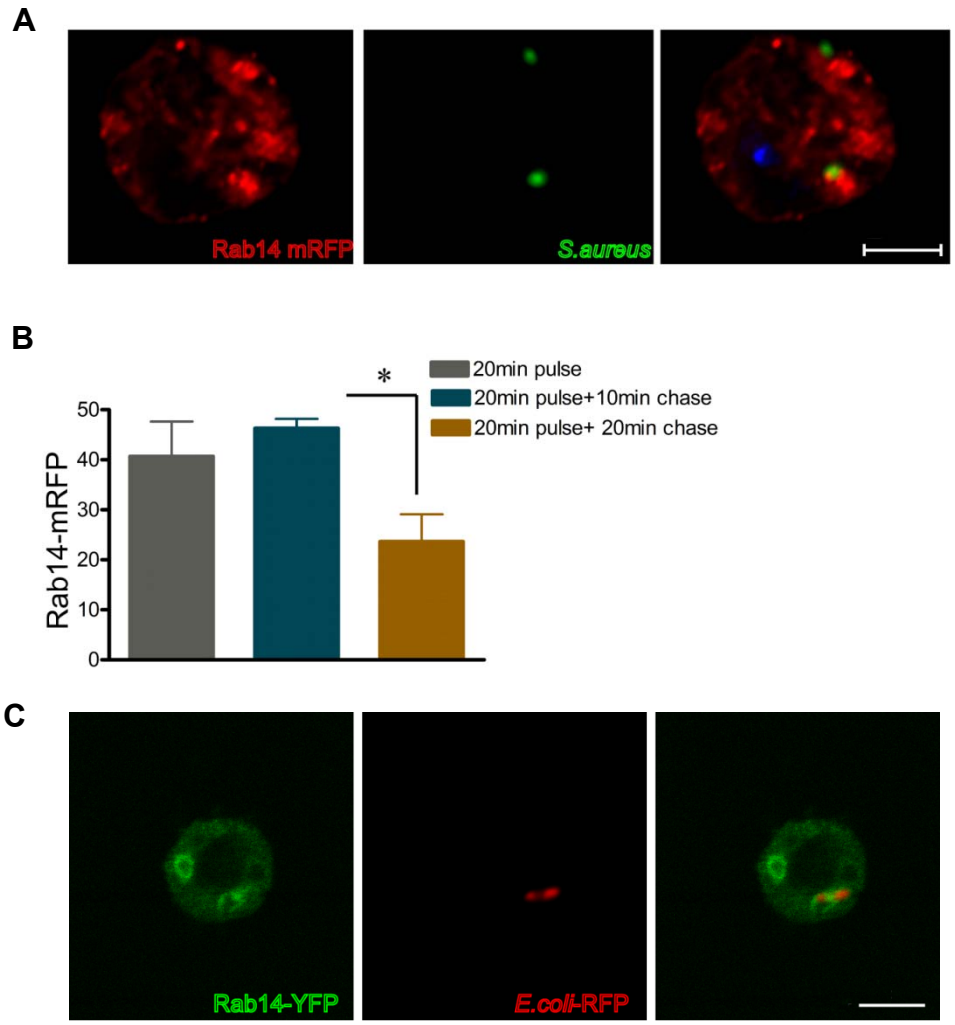

Figure 3-17. Rab14 is recruited to bacterial phagosomes (A) Larval hemocytes were bled out at different timepoints after infection with *S.aureus* conjugated to AF-488 (green). The phagosomes were evaluated for recruitment of Rab14 RB (red). A representative image from the 20 min pulse + 10 min chase is shown. (B) Percentage recruitment at different timepoints is plotted. (C) Larval hemocytes were bled after 20 min of infection with *E.coli* and show recruitment of Rab14 RA-YFP (green) onto the *E.coli* (red) phagosome. Error bars indicate standard error of the mean (SEM), n = 3. Scale bar, 5μm * $p \leq 0.05$.

To evaluate endocytosis in the fat body, we examined uptake and endosomal maturation using Texas red-conjugated avidin as a tracer in the fat body. The initial endocytic uptake after a pulse of 20 min was comparable in wildtype and *Rab14* mutants (Fig. 3-18). As endosomal maturation progresses, the endocytic vesicle matures and moves inwards towards perinuclear lysosomal compartments. In wildtype, after a chase of 60 min, most of the tracer was concentrated in the perinuclear region; this was not the case for the mutant (Fig.3-18). This suggests endosomal maturation is affected in the *Rab14* mutant.

We then looked at the expression of GFP-LAMP in the *Rab14* mutants. After 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* mutants accumulated significant GFP-LAMP perinuclear puncta, indicating a defect in trafficking (Fig.3-19). The simplest interpretation is that these results are consistent with defects in late endosome to lysosome trafficking.

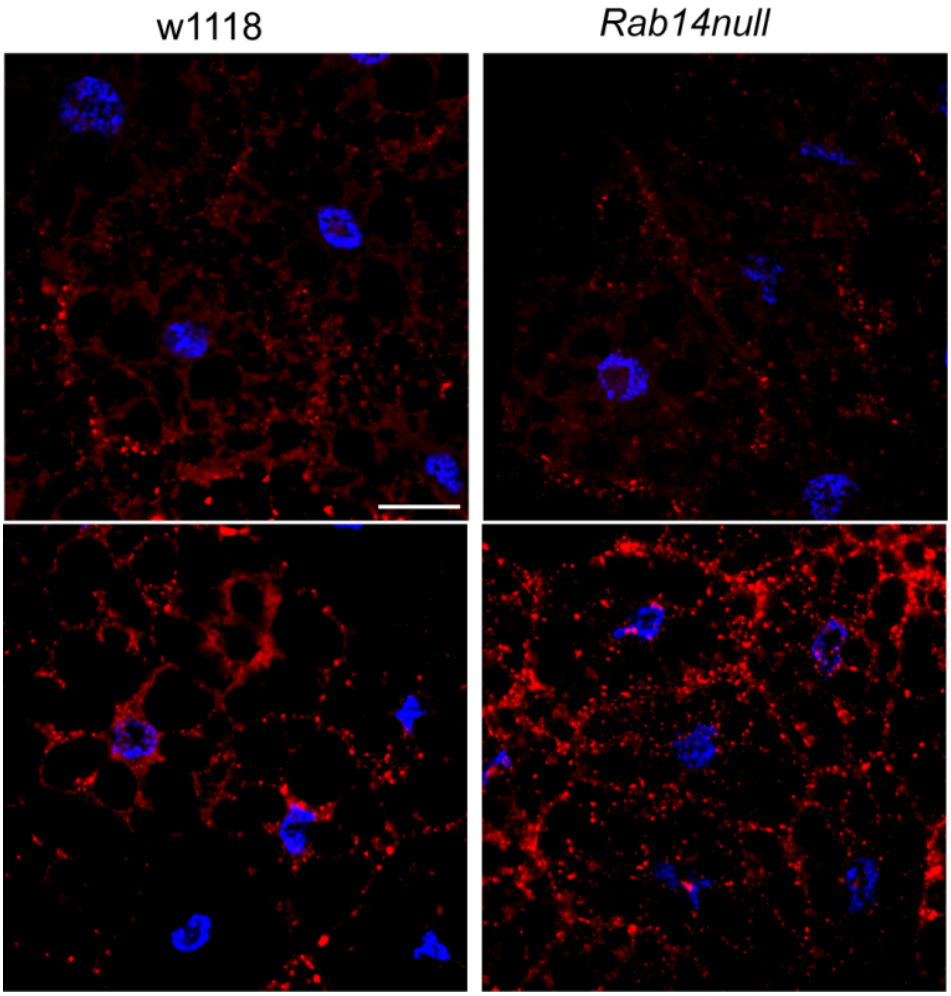

Figure 3-18. *Rab14* mutants show a defect in fluid phase endosomal maturation Fluid phase endocytosis and endosomal maturation assays with TR-Avidin in wildtype and *Rab14* mutants. Larval fat body was dissected out and examined after a pulse of 20 min with TR-Avidin (top panel) or with the pulse followed by a chase of 60 min (bottom panel). The experiment was repeated at least 3 times with ~ 15-20 larvae. Scale bar 20μm

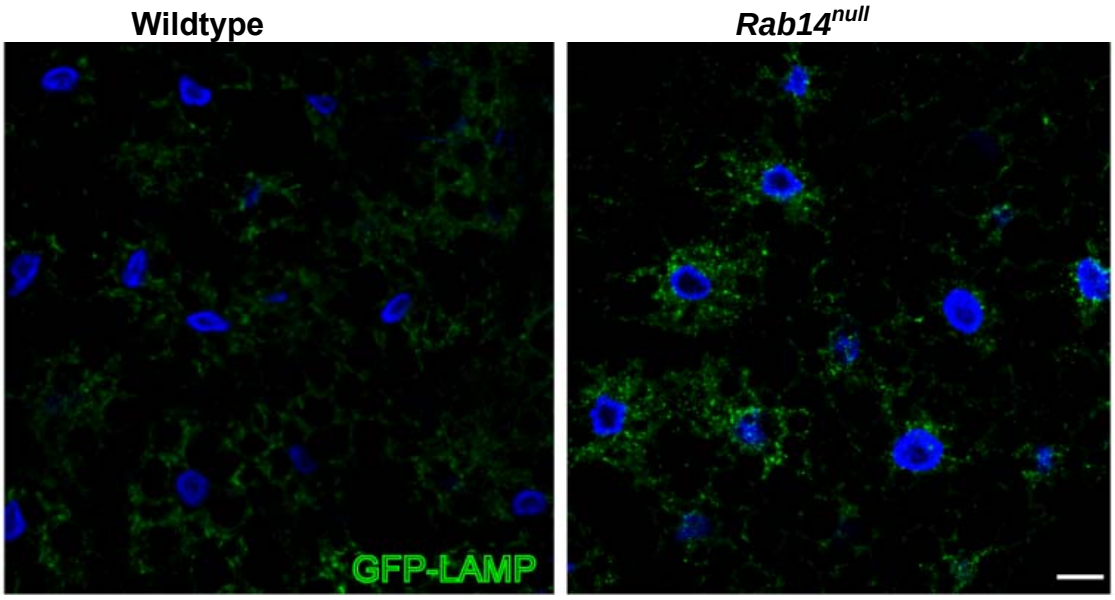

Figure 3-19. *Rab14* mutants show a defect in trafficking from late endosomes to lysosomes Fat body from wildtype and *Rab14* mutant larvae expressing tubulin > GFP-LAMP was dissected out and fixed. The experiment was repeated 3 times with ~ 15-20 larvae. Scale bar 20μm

Phagosome maturation is required to activate select humoral responses

The generation of a mutant affecting phagosome maturation allowed us to examine a relevant question: Is there crosstalk between the hemocyte-mediated cellular response and the fat body-mediated humoral response? We examined the induction of antimicrobial peptides in *Rab14* mutants in response to infection. Although, Drosomycin induction in response to *S.aureus* was comparable in wildtype and *Rab14* mutants (Fig.3-3A), there were significant reductions in Defensin and Cecropin induction (Fig.3-20A, B).

One possibility is that this reduced AMP induction could be due to the defect in phagosome maturation. Alternatively, a defect in endosomal maturation in the fat body might be required for an efficient AMP induction. Recently, endocytosis has been shown to be required for activation of the Toll pathway (Huang et al., 2010). To understand which tissue might be contributing towards the AMP phenotype, we inhibited the activity of endogenous Rab14 by expressing a Rab14DN in a tissue-specific manner.

We also examined a Rab5 down-regulated line to see whether low AMP induction is a Rab14-specific phenotype or a general result of defective endocytic or phagocytic machinery. Hemocyte-specific expression led to a significant decrease in Cecropin induction in Rab14DN but only a moderate decrease in the *Rab5* RNAi line (Fig.3-21A, B). However a similar reduction was not observed for Defensin expression, after downregulation of either of the two RabGTPases (Fig.3-22A, B). It is possible a more complete reduction in activity, as is achieved with the $Rab14^{null}$ mutant is required to influence

Defensin induction. The effect on Cecropin induction suggests that efficient phagosome maturation in the hemocyte may indirectly regulate AMP induction. On the other hand, fat body specific down-regulation of Rab5 or Rab14 activity led to decreases in both Cecropin (Fig.3-21A, B) and Defensin (Fig.3-22A, B) induction. Hence, endocytosis in the fat body also plays an important role in AMP induction. Hemocyte or fat body specific expression of Rab14 isoforms could partially rescue Cecropin expression suggesting that Rab14 function in both tissues is essential for an efficient induction (Fig. 3-20B). However for Defensin, induction could be completely rescued by Rab14 expression in either hemocytes or the fat body (Fig. 3-20A). The basal levels of Defensin expression is low and hence fold changes in expression are highly responsive to stimulus. This may be why a partial rescue for Defensin was not observed. This is also supported by the fact that down-regulation of Rab14 in hemocytes was sufficient to decrease Cecropin induction but not Defensin induction (Fig. 3-21A, 3-22A). Both Rab14 isoforms rescued the phenotype independently, confirming that they have similar function (3-20A, B). We propose that phagosome maturation regulates expression of specific AMPs, Cecropin and Defensin.

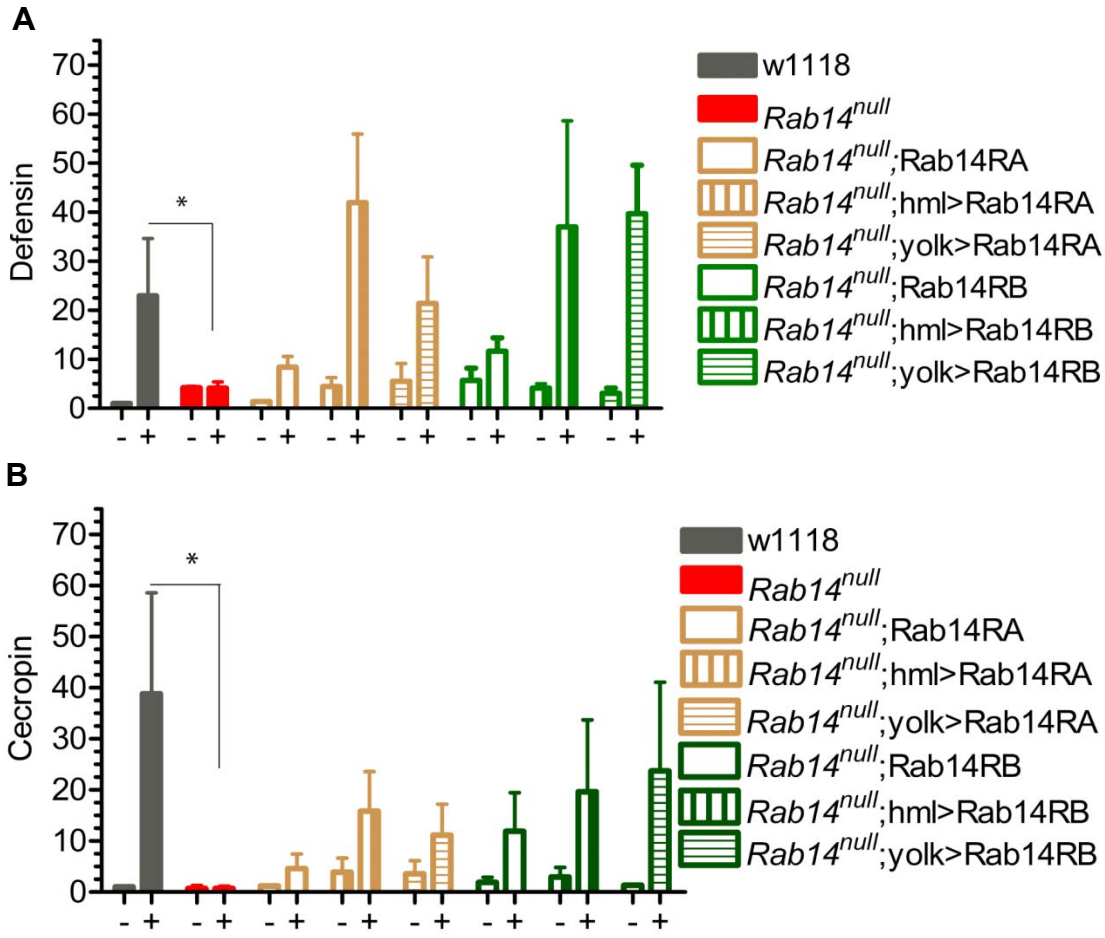

Figure 3-20. Rab14 is required for induction of Cecropin and Defensin

Wildtype and *Rab14* mutants were injected with concentrated log-phase culture of *S.aureus* and real-time PCR was done on samples collected after 6h to examine (A) Defensin and (B) Cecropin induction. The rescue lines were generated by hemocyte (hml) or fat body (yolk) specific expression of *Rab14* RA or RB in the *Rab14* mutant background. The line *Rab14*;Rab14RA/RB acts as a no driver control, – wounding control, +*S.aureus* infection. For each line, 7-10 female flies, aged 5-7 days were used. n = 3, error bars represent SEM. * $p \leq 0.05$.

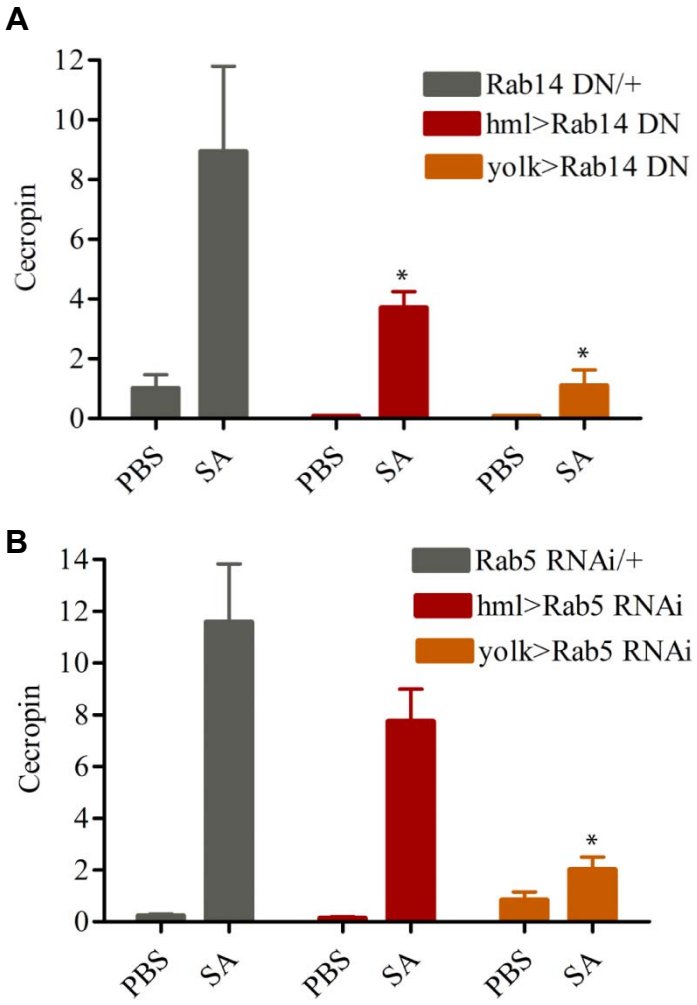

Figure 3-21. Endocytic machinery in the fat body and hemocytes are essential for Cecropin induction Cecropin relative expression after hemocyte (hmlGAL4) or fat body (yolkGAL4) specific down-regulation of (A) Rab14 (DN) or (B) Rab5 (RNAi) activity. The Rab14 DN or Rab5 RNAi line outcrossed to w1118 (the parental line for hmlGAL4) was used as a control. A concentrated log phase culture of *S.aureus* was injected and real time PCR analysis was carried out on samples collected after 6h. n = 3, error bars represent SEM. * $p \leq 0.05$.

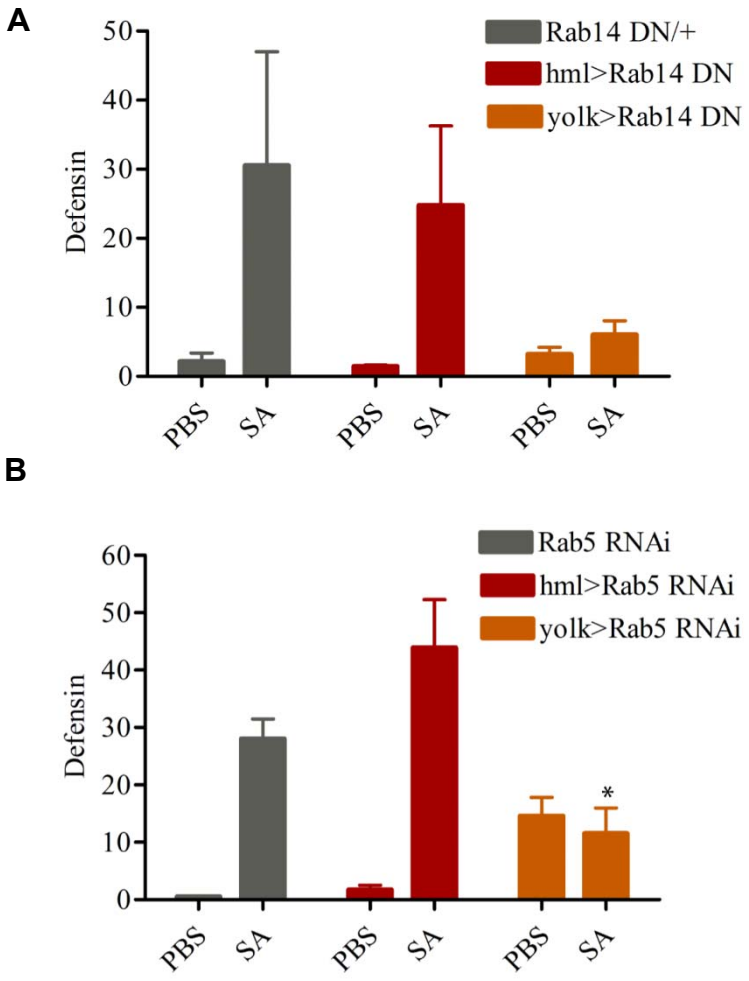

Figure 3-22. Endocytic machinery in fat body is essential for efficient defensin induction Defensin relative expression after hemocyte (hmlGAL4) or fat body (yolkGAL4) specific down-regulation of (A) Rab14 (DN) or (B) Rab5 (RNAi) activity. The Rab14 DN or Rab5 RNAi line outcrossed to w1118 (the parental line for hmlGAL4) was used as a control. A concentrated log phase culture of *S.aureus* was injected and real time PCR analysis was carried out on samples collected after 6h. n = 3, error bars represent SEM. * $p \leq 0.05$

Spatzle induction in hemocytes is affected in Rab14 mutants

We hypothesized that Rab14 function during phagosome maturation is essential for AMP induction. Functional ablation of hemocyte phagocytic activity using beads has been shown in Drosophila (Elrod-Erickson et al., 2000). We hypothesized lack of phagocytosis due to bead injection in flies should also reduce AMP induction. Contrary to our hypothesis, no differences in Cecropin induction between the control (water) injected or bead injected flies were detected (Fig. 3-23A), even though bead injection did result in reduction in phagocytosis (Fig. 3-23B). This is surprising but a similar observation had been reported earlier (Nehme et al., 2011). It is possible in the presence of bead-filled hemocytes, microbes become more persistent in the hemolymph resulting in increased contact with the fat body PRRs leading to expression of the AMPs. The bead injection led to a minor increase in Cecropin expression in *Rab14* mutants possibly due to the same reason.

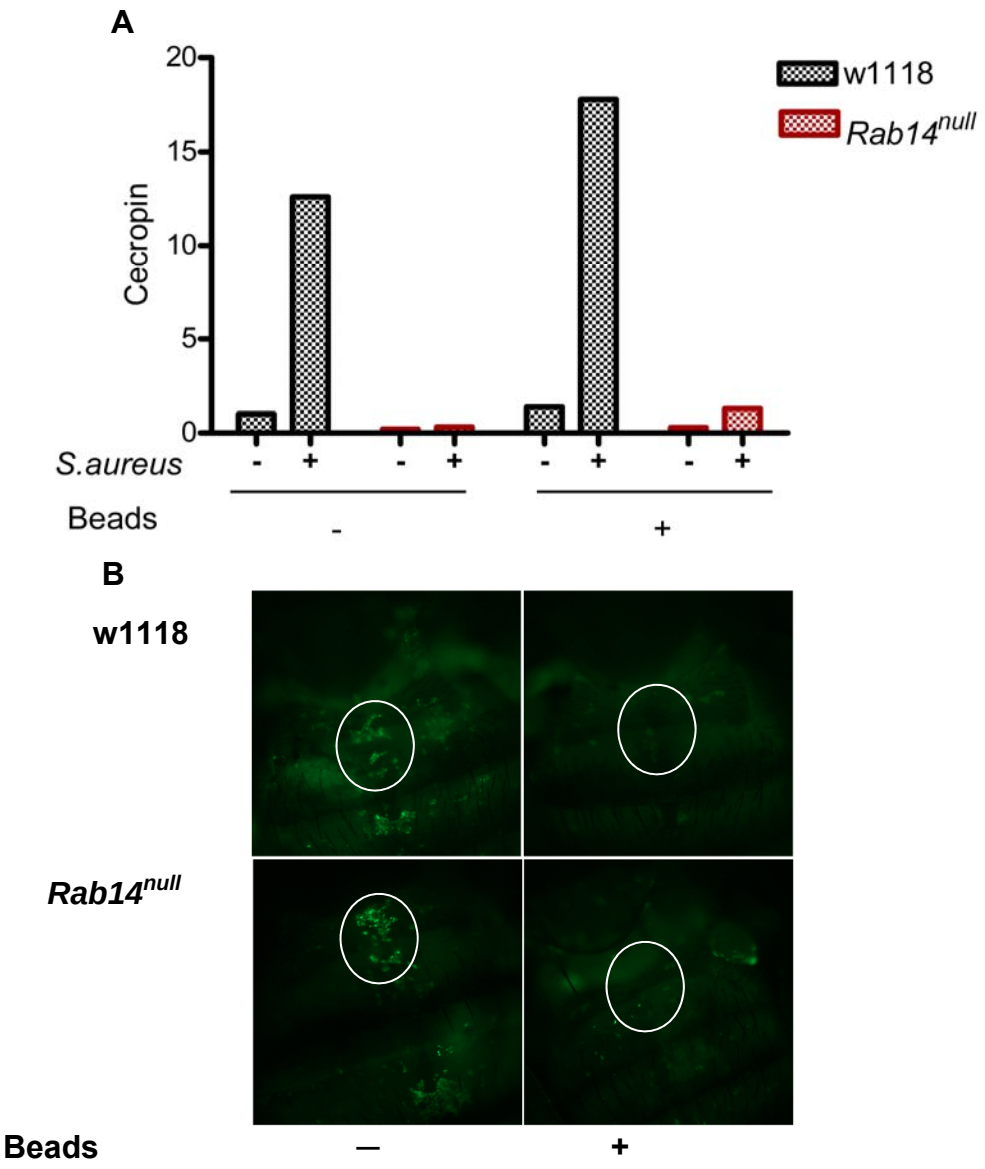

Figure 3-23. Bead-based functional ablation of hemocytes has no effect on Cecropin induction. (A) Cecropin induction in wildtype and *Rab14* mutants in response to *S.aureus* infection with or without bead induced functional ablation of hemocytes. (B) Wildtype and *Rab14* mutant flies examined for phagocytic uptake of fluorescein conjugated *S.aureus* before (-) or 24h after (+) bead injection

As the fat body is the primary source of AMP production, we examined if there was evidence of a cytokine being produced by the hemocytes to facilitate the cross talk with the fat body. Spatzle is the Toll ligand and has been proposed to be a cytokine that might regulate the humoral response (Schneider et al., 1994; Weber et al., 2003; Shia et al., 2009). To examine whether Rab14 regulates the humoral response through a cytokine, we looked at infection-induced induction of Spatzle (Fig.3-24). Wildtype flies induced Spatzle specifically in the hemocytes in response to *S.aureus* infection. *Rab14* mutants on the other hand, did not show a comparable induction in the hemocytes. However, levels of expression in the carcass were comparable in both wildtype and mutants. This suggests in response to an infection, Rab14 either through an independent role or due to its role in phagosome maturation result in Spatzle induction specifically in the hemocytes. Since the induction in the carcass was not affected, there may be an alternate pathway for Spatzle regulation in other tissues.

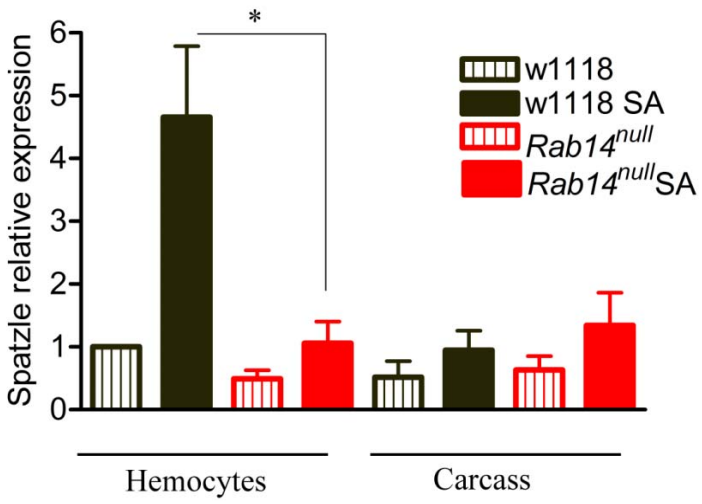

Figure 3-24. Rab14 is required for Spatzle induction in the hemocytes

Uninfected and *S.aureus* infected wild type and *Rab14* mutant larvae were bled out after 6 h to separate the hemocytes from the carcass. Spatzle relative expression was examined using Real-time PCR in both hemocytes and carcass samples. ~ 50 larvae were used for each line. The data is an average of three independent experiments. Error bars represents SEM. * $p \leq 0.05$.

Discussion

In previous studies, the removal (Defaye et al., 2009) or the functional disabling of hemocytes (Nehme et al., 2011) 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 varied means. Phagosome maturation in the hemocyte is essential for bacterial clearance. Along with that, hemocytes also limit susceptibility in a non-autonomous manner. A phagosome maturation mutant $Rab14^{null}$ demonstrated defective induction of Toll ligand Spatzle. Hence we propose, the phagocytic machinery signal to the fat body- mediated humoral response and regulates expression of specific AMPs which are effective against G+ bacteria.

RabGTPases are required for phagosome maturation (Vieira et al., 2002). We have identified Rab14 as an integral component of the endocytic/phagocytic machinery. *Rab14* mutants show defects in both *E.coli* and *S.aureus* phagosome maturation, signifying the crucial role that Rab14 plays during the process. However, we could not detect significant differences in Rab14 expression in response to infection in larvae. This could be due to regulation of activity mostly by the GTP/GDP switch. Furthermore, cell biological studies demonstrated that Rab14 is required for efficient recruitment of Rab7 onto the bacterial phagosome. This finding further broadens our understanding of maturation and points to a role for Rab14 during the Rab5 to Rab7 transition. In contrast to our findings, a prior study in

RAW cells 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). This might explain why characterization of a loss-of function mutant for Rab14 appears to give a very different answer as to its function.

A time line proteomic study of latex bead phagosomes indicated biphasic enrichment of Rab14 unlike the single phase enrichments of Rab5 and Rab7 onto the phagosomes (Rogers and Foster, 2007). 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 reflected in the observation that in the absence of Rab14, maturation is not completely abolished, but instead

displays slower kinetics. Also, the viability of *Rab14*^{null} is not compromised, while a more severe defect in endocytosis 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 and Scidmore, 2007). For instance, Rab5 (Kelley and Schorey, 2003) and Rab14 (Kyei et al., 2006) are actively retained for an extended period of time on mycobacterial phagosomes which do not mature into a 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 aiding maturation arrest. A Rab14-related RabGTPase, RabD regulates phagosome-phagosome fusion in *Dictyostelium,* (Harris and Cardelli, 2002). Homotypic phagosome fusion is unique to *Dictyostelium* phagosome maturation (Duhon and Cardelli, 2002). However in mammalian cells, intracellular pathogens promote homotypic phagosome fusion as a survival strategy (Brumell and Scidmore, 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.

The cellular response is activated quickly in response to infection (Haine et al., 2008), so it can potentially warn the host of incoming danger and activate the slower humoral response. However, the cellular response

and humoral immune responses show temporal (Haine et al., 2008) and spatial separation. The former takes place in hemocytes, while the latter is mostly mediated by the fat body. Psidin, a lysosomal protein has been shown to be associated with bacterial clearance and AMP induction (Brennan et al., 2007). As Psidin's function in the lysosome is not known, it was unclear how phagosome maturation regulated AMP induction. Our data is the first to demonstrate that Rab14 which plays an essential role during phagosome maturation in the hemocyte is essential for induction of the AMP genes Cecropin and Defensin. It is noteworthy that both of the affected AMPs, Cecropin and Defensin have strong antimicrobial activity against G+ bacteria (Samakovlis et al., 1990; Tzou et al., 2002). The upstream sequence of these two AMP genes have κB binding sites like the other AMPs, but they also have a unique upstream element R1 (Uvell and Engstrom, 2003). The presence of both the elements are essential for Cecropin induction, but the peak for R1 binding activity in the nucleus occurs sooner than the kB binding activity, suggesting that the R1 elements respond during the early phase of infection. It is possible that a cytokine, resulting from phagosome maturation in the hemocyte, signals to the fat body and leads to activation of the R1 binding protein. We demonstrate that *S.aureus* infection induces mRNA expression of the cytokine Spatzle in the hemocytes and that this is regulated by Rab14 function. Spatzle may be required for activation of the R1 binding factor following Toll signaling. However, the existence of another cytokine and/or signaling pathway to achieve the same cannot be ruled out.

Apart from the cellular response, endocytosis has also been proposed to regulate the Toll-mediated humoral response. Endosomal proteins were identified as regulators of the Toll pathway in a *Drosophila* S2 cell screen (Huang et al., 2010). Myopic (Mop) is an endosomal protein of the tyrosine phosphatase family and is required for Toll pathway activation in *Drosophila* S2 cells and adults. Genetic studies placed *myopic* upstream or in parallel to the adaptor MyD88, but downstream to Spatzle in the Toll signaling pathway. Another endosomal protein Hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs) was also shown to have a role similar to Myopic. Furthermore, Toll, Mop and Hrs were shown to exist in a complex, indicating that *Drosophila* Toll is also found in the endosomal compartment.

The exact contribution of these endosomal proteins toward Toll signaling is not well understood. Mammalian TLR4 signals from both the plasma membrane and early endosomes (Kagan et al., 2008), so it is possible that this function is conserved for Drosophila Toll. Although we did not observe a reduction in Drosomycin expression as previously noted, the expression of Cecropin and Defensin was significantly reduced when the endocytic pathway was disrupted in the fat body. Thus RabGTPases, due to their critical role during endosomal maturation also play an important role in the activation of Toll signaling. In mammals, uptake of a microbe by phagocytic cells leads to expression of cytokines (Medzhitov and Janeway, 1997). Interestingly, likewise in *Drosophila*, the cytokine Spatzle has been shown to be expressed in the hemocyte specifically in response to an

infection (Shia et al., 2009). We have identified Rab14 as a regulator of Spatzle expression during *S.aureus* infection. We propose that phagosome maturation signals Spatzle expression and an inefficient phagosome maturation in *Rab14* mutants results in defect in Spatzle expression. However it is also possible that Rab14 exerts its effect through an independent function. Since the function of the RabGTPases have been mostly conserved during evolution, it will be interesting to see whether Rab14 also regulates cytokine expression in mammals.

Lastly, there is a limited understanding of the process and function of phagosome maturation in *Drosophila*. Our work examines the process and underscores its role in limiting susceptibility to pathogenic *S.aureus*. Furthermore, Rab14 has been identified as a positive regulator of phagosome maturation critical for Rab7 recruitment. Rab14 is a target of intracellular pathogens like *Mycobacteria* (Kyei et al., 2006) and *Salmonella* (Kuijl et al., 2007). Another intracellular microbe *Helicobacter pylori* forms Rab7-positive megasomes which harbor multiple bacteria, as a result of homotypic phagosome fusion (Allen et al., 2000; Borlace et al., 2011). Since Rab14 localized to late phagosomes, it would be interesting to see if Rab14 plays a role in *H.pylori* pathogenesis. Rab14 has been found to be critical for the pathogenesis of different intracellular microbes. 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.

Experimental procedures

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:

Forward (For):5´-ACTGTGCGTTAGGTCCTGTTCA-3´

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: $Rab14^{EY04032}$, $Rab14^{BG01134}$, Rab14 RA-YFP, Rab14DN, Rab5RNAi, Rab7RNAi, hmlΔGAL4, cgGAL4, actinGAL4 were obtained from Bloomington stock center. Other lines Rab5-GFP, Rab7-GFP (Marcos Gonzáles-Gaitán), tubulin>GFP-LAMP (Helmut Kramer), Spinster-GFP (Graeme Davis) were generous contributions.

Adult phagocytosis and phagosome maturation experiments

Phagocytosis: 5-7 day old adult flies were injected with fluorescein-conjugated *E.coli* or *S.aureus* resuspended in PBS (Invitrogen: E2861, 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 stereo microscope (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.

Phagosome maturation: 5-7 day old adult flies were injected with pHrodo-conjugated *E.coli* or *S.aureus* resuspended in PBS (Invitrogen: P3536, A10010, 8mg/ml). pHrodo conjugates were stored in small aliquots at -20^{0}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.

Function ablation of hemocytes using beads: 5-7 day old adult flies were injected with 4X concentrated fluospheres (carboxylate modified microspheres 0.2µm, Invitrogen: F8805). Only water injection was used as control. The flies were assessed for phagocytic uptake or further injected with bacteria to examine AMP induction.

Phagosomal marker recruitment and colocalization experiments

Phagosomal marker recruitment: 8-10 wildtype and the *Rab14* mutant feeding 3rd instar larvae were injected with Alexa-fluor (AF) conjugated *S.aureus* (Invitrogen: S23371, S23372) or RFP expressing *E.coli* for the 20 min pulse. 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-specific drivers hmlΔGAL4 (Goto et al., 2001) or hemeseGAL4 (Kurucz 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 antibody labeled with AF-488 were used.

Co-localization experiments: Feeding third instar larvae expressing the tagged endosomal markers were injected with *E.coli* and 15 or 45 min later, hemocytes were bled, followed by fixing and mounting. Fat body from feeding 3rd instar larvae expressing the tagged endosomal markers using the cgGAL4 driver, were dissected out from larvae fixed and mounted.

Endosomal maturation

Texas Red (TR)-avidin endocytosis and maturation: The fat body from feeding 3rd instar larvae was dissected out in PBS and given a pulse of TR-Avidin (80µg/ml) in SM and incubated for 20 min. The tissue was rinsed two times and washed 3 times (over a period of 15 min) with SM + 10% FBS. This was followed by a chase of 45 min in SM + 10% FBS, after which tissue was fixed. The pulse, chase and washes were done at RT on a slow shaking nutator.

Image acquisition

Images were acquired using a Zeiss LSM 710/510 (100x/1.4 Oil Plan Apochomat lens or 40x/1.3 Oil Plan NeoFluar) or a Leica SP5X confocal (63X/1.4-0.6 Oil Plan Apochomat lens). Acquisition software Zen 2000 / LSM for Zeiss 710/510 and LAS-AF for Leica-SP5X 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 Real-time PCR

AMP induction: Adult flies 5-7 day old, were injected with PBS or a log phase culture of *S.aureus* (final OD 4.0) for the 6 h timepoint. For the 24 h timepoint, an overnight (O/N) culture of *S.aureus* (OD 4.0) was used. For the *E.coli* based experiments, an O/N culture was used. 8-10 flies were used for each experiment.

Spatzle induction: 40-50 feeding larvae were injected with an O/N culture of *S.aureus*. After 6 h, the hemocytes were bled out in cold PBS and collected, and the rest of the carcass was harvested.

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).

Primer Sequence

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´

Diptericin: For: 5'-CACGAGCCTCCATTCAGTCCAATCTCGTG-3',

Rev: 5'-TTTGCAGTCCAGGGTCACCA-3'

Drosomycin: For: 5'-CATCCTGAAGTGCTGGTGCGAAGGATG-3',

Rev: 5'-ACGTTCATGCTAATTGCTCATGG-3'.

CecropinA1 For: 5'-GACAATCCCACCCAGCTTCCGATTG-3',

Rev: 5'-TTTCGTCGCTCTCATTCTGG-3';

Defensin: For 5'-GACAAGAACGCAGACGGCCTTG-3',

Rev: 5'-CCACATGCGACCTACTCTCCA-3';

RP49: For:5'-CACGATAGCATACAGGCCCAAGATCGTG-3',

Rev:5'-GCCATTTGTGCGACAGCTTAG-3'

Spatzle: For:5'- GGAGCGGATCAACCCTGTG-3',

Rev: 5'-TTGGATTATAGCTCTGCGGAAAG-3'

Spatzle primer sequence was obtained from (Irving et al., 2005)

Survival and bacterial load experiments

Survival: Adult flies, 5-7 day old were injected with logarithmic phase culture of *S.aureus* (final OD 0.5) or *E.coli* (final OD 50) resuspended in PBS. Flies injected with PBS served as a wounding control. For *E.coli* survival, dead *E.coli* (80^{o}C for 1h) of OD 50 was used as a 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.

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.

Statistics

The Mann-Whitney test was used to calculate p-values. * $p \leq 0.05$, ** $p < 0.01$, *** $p < 0.001$. Log-rank tests were used to test for significant differences in survival curves, and p-values of < 0.05 were deemed significant.

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