ABSTRACT

Title of Dissertation:	GENOME WIDE ASSOCIATION STUDIES OF PHAGOCYTOSIS AND THE CELLULAR IMMUNE RESPONSE IN *DROSOPHILA MELANOGASTER*
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Phagocytosis of bacteria by specialized blood cells, known as hemocytes, is a vital component of *Drosophila* cellular immunity. To identify novel genes that mediate the cellular response to bacteria, we conducted three separate genetic screens using the Drosophila Genetic Reference Panel (DGRP). Adult DGRP lines were tested for the ability of their hemocytes to phagocytose the Gram-positive bacteria *Staphylococcus aureus* or the Gram-negative bacteria *Escherichia coli.* The DGRP lines were also screened for the ability of their hemocytes to clear *S. aureus* infection through the process of phagosome maturation. Genome-wide association analyses were performed to identify potentially relevant single nucleotide polymorphisms (SNPs) associated with the cellular immune phenotypes. The *S. aureus* phagosome maturation screen identified SNPs near or

in 528 candidate genes, many of which have no known role in immunity. Three genes, *dpr10, fred,* and *CG42673,* were identified whose loss-of-function in blood cells significantly impaired the innate immune response to *S. aureus.* The DGRP *S. aureus* screens identified variants in the gene, *Ataxin 2 Binding Protein-1* (*A2bp1)* as important for the cellular immune response to *S. aureus*. *A2bp1* belongs to the highly conserved Fox-1 family of RNA-binding proteins. Genetic studies revealed that *A2bp1* transcript levels must be tightly controlled for hemocytes to successfully phagocytose *S. aureus*. The transcriptome of infected and uninfected hemocytes from wild type and *A2bp1* mutant flies was analyzed and it was found that *A2bp1* negatively regulates the expression of the Immunoglobulin-superfamily member *Down syndrome adhesion molecule 4* (*Dscam4*). Silencing of *A2bp1* and *Dscam4* in hemocytes rescues the fly's immune response to *S. aureus* indicating that Dscam4 negatively regulates *S. aureus* phagocytosis. Overall, we present an examination of the cellular immune response to bacteria with the aim of identifying and characterizing roles for novel mediators of innate immunity in *Drosophila.* By screening panel of lines in which all genetic variants are known, we successfully identified a large set of candidate genes that could provide a basis for future studies of *Drosophila* cellular immunity. Finally, we describe a novel, immune-specific role for the highly conserved Fox-1 family member, *A2bp1*.

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by

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Dissertation submitted to the Faculty of the Graduate School of the
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Dedication

To my grandfather

my mother

my sisters

my husband

and my children, Isabel and Jonathan.

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Chapter 1

Introduction

I. Drosophila Innate Immunity

To combat infection, organisms rely on a multitude of immune defense mechanisms designed to recognize and eliminate invading microbes. The immune response can be functionally divided into two main classes: innate and adaptive immune responses. Adaptive immune responses are highly specific, take time to develop, and result in lifetime protective immunity to a particular pathogen. On the other hand, innate immune responses are available immediately to combat a wide array of pathogens. Innate and adaptive immune responses are complementary and both are necessary to produce robust immunity. Cells of the innate immune system serve as the initial line of defense against invading microbes. They express germline-encoded receptors that identify common pathogen associated molecular patterns (PAMPs) such as bacterial derived lipopolysaccharide (LPS), lipoteichoic acid (lipid anchored teichoic acids (LTA)), peptidoglycan, double-stranded RNA, and unmethylated CpG DNA or β-glucan of fungi. Innate immune cells eliminate most pathogenic microorganisms before they can cause disease. The importance of innate immunity is underscored by the fact that innate immune responses are found in nearly all animals, while adaptive immune responses are restricted to the jawed vertebrate group.

Phagocytosis is a cell mediated immune response to foreign matter. The process of phagocytosis was first described over 100 years ago by Èlie Metchnikoff (Kaufmann, 2008). It is a receptor-mediated event that occurs when pattern recognition receptors (PRRs) on the surface of the cell recognize and bind to ligands on target molecules (Flannagan et al., 2012). Once bound to their cognate ligands, these receptors initiate signaling events that lead to the clearance of pathogens. Phagocytosis is the cornerstone of a robust and powerful innate immune response. In mammals, specialized blood cells, macrophages, neutrophils and dendritic cells, take up microbes and destroy them within the cell. Some innate immune cells also serve as professional antigen presenting cells, a role that is critical for the activation of adaptive immune effector cells such as B and T lymphocytes.

Investigators researching mammalian phagocytes have utilized *ex vivo* and *in vitro* cell biology and microscopy techniques to examine the molecular mechanisms underlying phagocytosis. Individual phagocytic components have been studied through the use of non-phagocytic cell lines that exogenously express phagocytic receptors or in primary cells isolated from mutant mice (Stuart and Ezekowitz, 2005). The most extensively studied mammalian phagocytic receptors are the Fc receptor (FcR) and the complement receptor, CR3 (Griffin et al., 1975; Odin et al., 1991). These receptors are regarded as a general model for the cellular and molecular events that take place during phagocytosis. While these reductionist approaches have increased our understanding of the complex cell biology of phagocytosis, they do not address the relative importance of the cellular immune response in intact organisms. Genetically tractable model systems such as the nematode, *Caenorhabditis elegans,* and the fruit fly, *Drosophila*

melanogaster, have been successfully used to study host-pathogen interactions *in vivo*. In particular, work defining the components that mediate the cellular immune response has greatly enhanced our understanding of the significant immune and homeostatic roles played by phagocytic cells *in vivo*.

Drosophila melanogaster relies on innate immunity to defend against attacks by parasites, fungi, viruses and bacteria (Lemaitre and Hoffmann, 2007). Many *Drosophila* genes and signaling pathways are conserved in higher organisms and studies of fruit fly immunity have provided valuable insight into human innate immune responses. The defense reactions of *Drosophila* include potent humoral and cellular responses. Humoral immunity is characterized by the systemic production of antimicrobial peptides (AMPs) after immune cells of the blood and fat body detect bacteria or fungi in the hemolymph. The cellular immune response is specifically carried out by specialized blood cells known as hemocytes, which engulf and eliminate pathogens via phagocytosis.

II. The Cellular Immune Response

There are three classes of hemocytes in the fly: plasmatocytes (90-95% of blood cells), crystal cells (5% of blood cells) and lamellocytes. The plasmatocytes are professional phagocytes and are similar to the mammalian macrophages (Williams, 2007). During development plasmatocytes, also called pupal macrophages, ingest apoptotic cells and larval tissues (Tepass et al., 1994). After metamorphosis, plasmatocytes are the only hemocyte lineage present, with an estimated 1000-2000 plasmatocytes present at the adult stage (Lanot et al., 2001). Many of these plasmatocytes adhere to the heart tissue in the dorsal vessel where they function as the primary effector of cellular immunity in adults (Elrod-Erickson et al., 2000). Survival

experiments carried out using flies where plasmatocytes were genetically ablated show that phagocytosis is critical in the clearance of the Gram-positive bacteria, *Enterococcus faecalis* and *Staphylococcus aureus* (Charroux and Royet, 2009; Defaye et al., 2009; Nehme et al., 2011).

Crystal cells make up 5% of circulating hemocytes in embroyos and larvae. These cells mediate melanization reactions by releasing the prophenoloxidases (PPOs), PPO2 and PPO3, after injury (Dudzic et al., 2015; Kurucz et al., 2007b). Phenoloxidase (PO) is a key enzyme in melanin biosynthesis and it is synthesized when its precursor, the zymogen PPO is cleaved to generate active PO (Tang, 2009). The lamellocytes can be induced upon pupariation, but also differentiate in larvae in response to parasitic wasp infections. Lamellocytes encapsulate parasitic wasp eggs deposited in the larval hemocoel and release PPO3 to melanize the eggs (Dudzic et al., 2015; Rizki and Rizki, 1992).

A. Phagocytosis

Phagocytosis is initiated when cell surface receptors recognize their target ligands and trigger engulfment of molecules into a nascent organelle, the phagosome. Phagocytic receptors can either directly bind to ligands expressed on the surface of target cells or recognize targets coated by opsonins, soluble host factors that bind to foreign bodies. Many phagocytic receptors are able to recognize multiple microbial and apoptotic cell-associated ligands. Additionally, due to the inherent diversity of particles that are taken up by phagocytosis, multiple receptors are simultaneously engaged to ligands on the surface of target particles to facilitate uptake. The overlap and redundancy in receptor ligand specificities helps in the formation of strong interactions between the target

particle and the phagocyte. Receptor redundancy is also evolutionarily advantageous as it allows the host cell to combat pathogens that have developed mechanisms to evade detection by a particular receptor.

Phagocytosis is broadly defined as the ingestion of large particles (≥0.5 μM), such as microorganisms or apoptotic cells. The cellular events that occur throughout the process can be separated into several stages: 1) particle recognition and binding 2) particle engulfment 3) phagosome maturation and pathogen degradation. The remainder of this section will discuss what is known and what is unknown about phagocytosis in *Drosophila* within the context of the schematic in Figure 1-1.

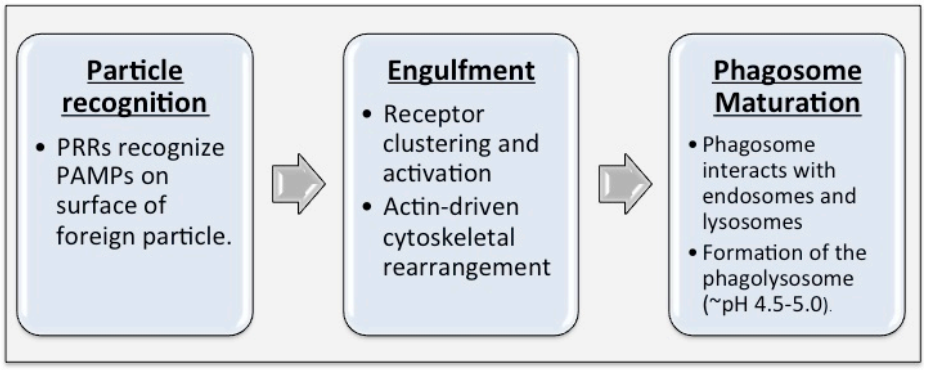

Figure 1-1: Stages of phagocytosis and phagosome maturation during the innate immune response.

Phagocytosis is initiated when pattern recognition receptors (PRRs) on the surface of phagocytic cells recognize and bind to pathogen associated molecular patterns (PAMPs) located on the surface of microbes. Once bound by cognate ligands, the receptors are activated, leading to the formation of signaling cascades that cause the actin cytoskeleton to rearrange, facilitating particle uptake into a de-novo organelle, the phagosome. The phagosome undergoes a series of coordinated fusion-fission events with endosomal vesicles during the process of phagosome maturation. Phagosome maturation culminates in the formation of the highly acidic and microbicidal phagolysosome.

Table 1: Cell surface recognition receptors and opsonins

Receptor Family	Receptor	Ligands and References	Related mammalian molecules
Scavenger receptors	Croquemort	Apoptotic cells (Franc et al., 1999) *Staphylococcus aureus* (Stuart et al., 2005)	CD36
	Peste	*Mycobacterium fortuitum* (Agaisse et al., 2005; Philips et al., 2005) *Mycobacterium smegmatis* (Philips et al., 2005) *Listeria monocytogenes* (Agaisse et al., 2005) NOT *Escherichia coli* or *S. aureus* (Philips et al., 2005)	SCARB1
	SR-C1	*E. coli* (Ramet et al., 2001) *S. aureus* (Ramet et al., 2001) NOT *Candida salvatica* (Ramet et al., 2001) Double stranded RNA (Ulvila et al., 2006)	None found
Nimrod receptor superfamily	Eater	*E. coli, Serratia marcescens,* and *S. aureus* (Chung and Kocks, 2011; Kocks et al., 2005) *Enterococcus faecalis* (Chung and Kocks, 2011; Nehme et al., 2011) NOT *Micrococcus luteus* (Chung and Kocks, 2011; Nehme et al., 2011) Double stranded RNA (Ulvila et al., 2006)	SREC; MEGF10; MEGF11; CD91; Stabilin 1 & Stabilin 2
	NimC1	*E. coli* (Kurucz et al., 2007a) *S. aureus* (Kurucz et al., 2007a)	
	Draper	Apoptotic cells (Freeman et al., 2003; Manaka et al., 2004) Axon pruning (Awasaki et al., 2006; MacDonald et al., 2006) *S. aureus* (Shiratsuchi et al., 2012)	
Peptidoglycan recognition proteins	PGRP-LC	*E. coli* (Bergeret et al., 2008; Ramet et al., 2002) NOT *S. aureus* (Ramet et al., 2002) NOT *E. coli* or *S. aureus* (Choe et al., 2002; Garver et al., 2006)	Mammalian PGRPs
	PGRP-SC1A/picky	*S. aureus* (Garver et al., 2006) NOT *E. coli* or *Saccharomyces cerevisiae* (Garver et al., 2006)	
	PGRP-SA	*S. aureus* (Garver et al., 2006) NOT *E. coli* (Garver et al., 2006)	

Integrins	Integrins αPS3/βν	Apoptotic cells (Nagaosa et al., 2011; Nonaka et al., 2013) *S. aureus* (Nonaka et al., 2013; Shiratsuchi et al., 2012)	ITGA4 (CD49D) ITGB1
Ig-like	Dscam	*E. coli* (Dong et al., 2006; Watson et al., 2005) *S. aureus (Dong et al., 2006)*	DSCAM
TEPs	Mcr (TEPVI)	*Candida albicans* (Stroschein-Stevenson et al., 2006)	
	TEP II	*E. coli* (Stroschein-Stevenson et al., 2006)	Complement components
	TEP III	*S. aureus* (Stroschein-Stevenson et al., 2006)	

Table 1-1: Cell surface recognition receptors and opsonins.

Drosophila phagocytic receptors and mammalian orthologs are grouped according functional properties and common features. EGF-like-repeat containing protein orthologs were identified in Kocks *et al,* 2005. All other mammalian orthologs, were identified using the Drosophila RNAi Screening Center (DRSC) Integrative Ortholog Prediction Tool (DIOPST) (Hu et al., 2011). CD36, CD36 (thrombospondin receptor); SCARB1, scavenger receptor class B, member 1; SR-C1, scavenger receptor class C, type 1; αPS3, integrin alpha PS3 subunit (encoded by *scab*); βν, integrin beta subunit (encoded by *Itgbn*); Ig-like, Immunoglobulin-like; Dscam, Down syndrome cell adhesion molecule; TEP, Thioester-containing protein.

B. Particle Recognition: The Receptors

The fruit fly *Drosophila melanogaster* is a model organism that has been successfully utilized to identify several pattern recognition receptors via large-scale RNA interference screens and smaller, classical genetic screens. A brief description of the

phagocytic receptors identified in *Drosophila* is given in Table 1-1 and in the accompanying text.

Recognition of Apoptotic Cells:

Phagocytic cells target two main classes of particles: apoptotic cells and microorganisms. Removal of apoptotic cells is key during embryogenesis and development (Arandjelovic and Ravichandran, 2015). Apoptotic cells display "eat me" signals. The most extensively characterized eat me signal is phosphatidylserine (PtdSer), a membrane lipid found on the inner leaflet of the plasma membrane in healthy cells (Fadok et al., 1992). Receptor ligation with PtdSer triggers phagocytic uptake of apoptotic cells in mice, humans, *Danio renio* (Zebrafish), *Caenorhabditis elegans* (nematodes) and *Drosophila* (Fadok et al., 1992; Hong et al., 2004; Li et al., 2003; Tung et al., 2013; Wang et al., 2003).

In mammals, removal of apoptotic cells is important for maintenance of routine tissue homeostasis and phagocytosis of apoptotic cell corpses is associated anti-inflammatory cellular responses (Poon et al., 2014). In *Drosophila,* three apoptotic cell receptors have been characterized: the CD36-related Scavenger receptor Croquemort (Crq), the EGF-like repeat-containing Nimrod family member Draper, and the integrin αPS3/βv (Franc et al., 1999; Manaka et al., 2004; Nagaosa et al., 2011) (Table 1). Of these three receptors, only Draper has been shown to directly bind PtdSer (Tung et al., 2013). Interestingly, Draper is a multivalent receptor and can recognize apoptotic cells by binding to PtdSer and/or the endoplasmic reticulum protein Pretaporter (Kuraishi et al., 2009)

Microbial receptors: Receptors that recognize PAMPs

Both professional phagocytic cells and non-professional phagocytic cells, such as endothelial and epidermal cells, are able to phagocytose invasive bacteria. Mammalian professional phagocytes are specialized blood cells: macrophages, neutrophils and dendritic cells. These cells respond to infection by migrating towards infected tissue. In *Drosophila* larvae, plasmatocytes are the primary phagocytic immune cells, and these cells also migrate to sites of infection. In *Drosophila* adults, plasmatocytes do not freely circulate, but mostly adhere to adult tissues such as the dorsal vessel (Elrod-Erickson et al., 2000; Lanot et al., 2001).

Detection of PAMPs on the surface of microorganisms is the first step in phagocytosis of commensal and pathogenic microbes. Receptors present on the extracellular side of the plasma membrane of phagocytes directly bind to the microbes or to opsonins that are deposited on the microbes' surface. Some of the receptors that participate in phagocytosis in *Drosophila* have mammalian orthologs with similar functions, while others are unique to insects. There are six main classes of molecules involved in pathogen recognition in the fruit fly: scavenger receptors, EGF-like-repeat containing Nimrod proteins, peptidoglycan recognition proteins, integrins, immunoglobulin-like proteins, and thioester-containing proteins.

Scavenger receptors:

Scavenger receptors are a group of structurally unrelated receptors with shared functional properties that bind multiple polyanionic ligands (Canton et al., 2013). The

receptors have heterogeneous structures and are subdivided into 9 classes (Class A-Class I) based on shared domain architecture.

A common feature of scavenger receptors is that they exhibit broad ligand specificity. For example, the mammalian Class B scavenger receptor CD36 recognizes altered self-ligands, acetylated and/or oxidized low density lipoprotein (LDL) and phosphatidylserine, as well as conserved microbial PAMPs from Gram-negative and Gram-positive bacteria.

During insect embryogenesis, hemocytes differentiate into macrophages that are able to phagocytose apoptotic cells (Tepass et al., 1994). Based on this observation, Ezekowitz and group (Franc et al., 1996) identified the Class B scavenger receptor, Croquemort. Using immunohistochemistry, they found Croquemort was expressed on hemocytes in embryogenesis and that this expression coincided with the developmental stage 11, when embryonic hemocytes developed the ability to phagocytose apoptotic cell corpses. The group also observed that Croquemort-positive hemocytes contained apoptotic cell corpses. Finally, transfecting non-phagocytic mammalian COS-7 cells transfected with Croquemort cDNA allowed these cells to bind to apoptotic cells *in vitro.* Genetic follow-up studies by the Ezekowitz group (Franc et al., 1999), using *croquemort* null flies, revealed that Croquemort is essential for phagocytosis of apoptotic cells *in vivo*. In 2005, Moore and colleagues (Stuart et al., 2005) found that Croquemort is also a receptor for *S. aureus,* but not *E. coli*, in a forward genetic screen using RNAi in S2 cells. Because Croquemort is a paralog of mammalian CD36, the group transfected human embryonic kidney (HEK) 293T cells with murine CD36 and assessed the ability of these cells to phagocytose heat-killed *S. aureus* and *E. coli.* They found that *crq* transfected

HEK293T cells were able to bind and internalize *S. aureus* and *E. coli,* with a three-fold increase in binding of *S. aureus* versus a two-fold increase for *E. coli* indicating that Croquemort has a higher affinity for the Gram-positive bacteria. Finally, macrophages from CD36 null mice showed impaired phagocytosis of *S. aureus* and LTA, and this defect was accompanied by a 60-70% reduction in the expression of the pro-inflammatory cytokines Tumor necrosis factor alpha (TNFα) and interleukin-12 (IL-12). The authors suggested that in addition to mediating phagocytosis of *S. aureus* and LTA, CD36 works with Toll-like receptors (TLRs) to initiate the cytokine response. They found that carboxyl terminal cytoplasmic domain of CD36 is required to trigger internalization of *S. aureus.* This domain also cooperates with TLR2/TLR6 to induce cytokine production through NFκB activation.

Class C scavenger receptors are only found in *Drosophila* species. One member, SR-CI, is an important phagocytic receptor in *Drosophila melanogaster*. In embryos, SR-CI is expressed in macrophages and when expressed in mammalian CHO cells, SR-CI exhibited high binding affinity for low density lipoprotein (Pearson et al., 1995). In 2001, the Ezekowitz group (Ramet et al., 2001) identified SR-CI as a receptor for Gram-negative and Gram-positive bacteria. Notably, the authors established an *in vitro* insect cell model to study phagocytosis. They compared the phagocytic potential of hemocytes and S2 cells, a primary cell culture derived from late stage *Drosophila* embryos, and found that both cells types efficiently phagocytose bacteria and yeast. They also examined whether *E. coli* and *S. aureus* were recognized by the same or different S2 cell receptors by performing cross-competition experiments. To do so, the authors coincubated S2 cells with unlabeled and fluorescently-labeled bacteria. Unlabeled *E. coli*

was able to decrease the amount of phagocytosed fluorescently-labeled *S. aureus,* and vice versa, suggesting the existence of common PRR(s) for *S. aureus* and *E. coli.* Interestingly, neither *E. coli* nor *S. aureus* inhibited the association of fluorescently-labeled yeast, *Candida silvatica*, indicating that the receptors for *C. silvatica* do not overlap with those for *E. coli* or *S. aureus.* Acetylated LDL, LTA, and polyinosinic acid inhibited the binding of both *E. coli* and *S. aureus* in a dose-dependent manner. The inhibition profile and bacteria phagocytosis profiles were similar to both the mammalian class A SRs and the binding characteristics for *Drosophila* SR-CI (Pearson et al., 1995). There are four members of the Class C SR family in Drosophila, dSR-CI, CII, CIII and CIV. SR-CI and CII are membrane bound receptors while CIII and CIV are predicted to encode secreted proteins. RNAseq analysis shows that SR-CIII and SR-CIV are expressed at low levels in S2 cells (Graveley et al., 2011). Conditioned media from S2 cells did not affect phagocytosis of bacteria, suggesting that neither CIII nor CIV played a role in the process of phagocytosis in this system. Additionally, the tissue and temporal expression analysis showed that SR-CI is expressed in larval hemocytes and throughout the life of the fly whereas the other Class C SR are only expressed in the early stages of development. The expression analysis, coupled with binding profiles for SR-CI indicated that it was a potential PRR candidate. *In vitro* binding experiments with SR-CI transfected CHO cells, found that SR-C1 acted as a receptor for Gram-negative (*E. coli*) and Gram-positive (*S. aureus*) bacteria, but not yeast (*Candida silvatica*). The authors also abolished the expression of *SR-C1* using dsRNA in the macrophage-like insect S2 cell line and observed a 20 and 30% reduction in the association of *E. coli* and *S. aureus,* respectively. This rather modest reduction in phagocytosis suggested that there must be

more than one receptor involved in the recognition of bacteria, and spurred future studies to identify additional PRRs in *Drosophila.* Finally, natural polymorphisms in *SR-CI* are associated with varying levels of resistance to the Gram-negative entomopathogen *Serratia marcescens,* indicating that SC-RI plays an important role in the immune response among wild fruit flies (Lazzaro, 2005; Lazzaro et al., 2004).

Mycobacterium marinum causes a lethal infection in *Drosophila* and during the early stages of infection the bacteria grows in phagocytes (Dionne et al., 2003). To identify potential receptors for *M. fortuitum*, a human pathogen, the Perrimon group conducted a genome-wide RNAi screen in S2 cells and identified a CD36 homolog, class B scavenger receptor Peste (Philips et al., 2005). The group treated S2 cells with dsRNA to deplete specific host genes, and then infected the cells with *M. fortuitum* that expressed GFP under the control of the *map24* promoter. The *map24* is responsive to low pH, such as the pH the internalized bacteria encounter in the lumen of the phagosome. In the screen, diminished GFP signal could occur from altered bacteria uptake, intracellular growth, or induction from the *map24* promoter. Silencing of *peste* in S2 cells blocked infection by *M. fortuitum*, but did not affect uptake of *S. aureus* or *E. coli.* Peste was also required for the uptake of the non-pathogenic *Mycobacterium smegmatis*, suggesting that Peste is a PRR for *Mycobacteria* species. Human embryonic kidney (HEK) 293 cells, which are normally refractory to infection by *M. fortuitum* could be infected when *peste* was heterologously expressed. Interestingly, heterologous expression of Peste in HEK293 cells caused a small increase in the uptake of *S. aureus* and *E. coli*. This result was not seen in experiments of silencing Peste in S2 cells. The authors suggest that the discrepancy could be explained by genetic redundancy provided by the presence of

multiple receptors for *S. aureus* and *E. coli* on the surface of S2 cells. Another S2 cell RNAi screen by the Perrimon group found that Peste is a receptor for another intracellular bacteria, *Listeria monocytogenes* (Agaisse et al., 2005). Both *M. fortuitum* and *L. monocytogenes* grow in *Drosophila* hemocytes (Dionne et al., 2003; Mansfield et al., 2003). It is possible that Peste has evolved to detect some component shared by *Mycobacteria* and *Listeria.* Both *Mycobacteria* and *Listeria* are lethal to the fly and both the bacteria replicate quickly within hemocytes and it is possible that these intracellular microbes enter hemocytes through interactions with Peste in order to establish a replicative niche within the phagosome of the cells.

Scavenger receptors are prominently expressed on the surface of mammalian macrophages and in *Drosophila* hemocytes. In both mammals and flies, SRs recognize altered self and microbial ligands and play essential roles in tissue homeostasis and immunity. In the fruit fly, Class B and Class C scavenger receptors have been shown to bind multiple self and non-self ligands to mediate phagocytosis of target particles. The work described above provides insight into the evolutionarily conserved role for scavenger receptors as important PRR involved in the innate cellular immune response in metazoans.

Nimrod receptor superfamily:

The Nimrod superfamily is a diverse class of proteins characterized by the presence of epidermal growth factor (EGF)-like repeats called NIM repeats (Kurucz et al., 2007a). Typical EGF repeats have roles in extracellular adhesion, coagulation and receptor-ligand interactions. The NIM repeat, also known as an EGF-like repeat, is a special type of the EGF domain that is shifted one cysteine unit compared to the typical

EGF repeat. All Nimrod superfamily proteins contain a signal peptide followed by N-terminal motifs of various kinds. Based on shared structural characteristics, the family is divided into three types: (1) Draper-type genes (Drosophila *nimrod A* and *Draper*) and the proteins containing many NIM subgroups (poly-NIM proteins), (2) Nimrod B-types (Drosophila *nimrod B 1-5*) and (3) Nimrod C-types (Drosophila *nimrod C 1-4* and *eater*) (Somogyi et al., 2008). The Draper-type group have an EMI domain (which may facilitate protein:protein interactions) at the N-terminal and one copy of the NIM motif followed by several EGF domains. Members of this subfamily have a wide distribution and are found in *C. elegans*, humans, and *Drosophila melanogaster*. Poly-NIM proteins have only been found in insects thus far. Nimrod-C family genes such as *eater* are transmembrane proteins with a variable number of NIM repeats. The Nimrod-B genes lack transmembrane domains and are most likely secreted proteins (Kurucz et al., 2007a).

The receptors for *E. coli* and *S. aureus* overlap in S2 cells. Silencing SR-CI only produces a modest reduction in phagocytosis of both types of bacteria, pointing to the existence of additional receptors for the bacteria. The GATA transcription factor Serpent regulates bacterial surface binding and the Ezekowitz group performed a microarray to identify potential receptors whose expression was regulated by Serpent in S2 cells (Kocks et al., 2005; Ramet et al., 2002). Forty-six of the genes down-regulated over 2-fold after *serpent* RNAi had signal sequences and transmembrane domains. They tested for effects on phagocytosis by silencing these candidates using RNAi in hemocytes and looking for binding of *S. aureus* and *E. coli* to the cells. One gene, which the researchers named *eater*, encodes a predicted cell-surface receptor and showed strong reduction in *S. aureus* and *E. coli* phagocytosis. The Eater protein contains an N-terminal signal peptide, 32

EGF-like domains, a transmembrane domain, and an intracellular C-terminal domain with a predicted tyrosine phosphorylation motif. The Eater protein has a low level homology (25% amino acid identity overall) to *C. elegans* CED-1, a receptor for apoptotic cells. The extracellular domain of CED-1 is homologous to a human scavenger receptor on endothelial cells, SREC. Analysis of *eater* expression showed that it is restricted to the plasmatocyte lineage.

The first four EGF-like repeats of Eater have a high level of amino acid diversity, suggesting that the N-terminal part of the protein may be important for binding to ligands. Amino acids 1-199 strongly bind to the Gram-negative bacteria *Serratia marcescens* and the Gram-positive bacteria *S. aureus*. Larval hemocytes from *eater* null flies showed significantly impaired phagocytosis of both *S. marcescens* and *S. aureus*. Adult *eater* null hemocytes were significantly impaired for phagocytosis of both *E. coli* and *S. aureus*. The researchers tested survival phenotypes of *eater* null flies by feeding flies the entomopathogen *S. marcescens,* thereby assessing the effects of phagocytic defects in a natural infection model. *eater* null flies were more susceptible to *S. marcescens,* and this increased susceptibility was accompanied by 10,000 fold higher levels of *S. marcescens* in the fly hemolymph. The antimicrobial peptides *Drosomycin* and *Diptericin* were induced normally in *eater* null mutants indicating that the susceptibility to *S. marcescens* was not attributed to defects in the humoral immune response but instead were likely caused by the impaired cellular immune response.

Follow-up studies utilizing a soluble Fc-tagged receptor variant of Eater comprised of the N-terminal 199 amino acids (Eater-Fc) revealed that Eater effectively binds to live or inactivated Gram-positive bacteria, *S. aureus* and *Enterococcus faecalis*

(Chung and Kocks, 2011). In contrast, Eater-Fc was unable to bind live or heat killed Gram-negative bacteria *E. coli, S. marcescens* and *Pseudomonas aeruginosa*. In order to recognize Gram-negative bacteria Eater-Fc required membrane-disrupting treatments. Accordingly, Eater-Fc bound Gram-negative ligands unmasked by treating the bacteria with the cationic AMP, Cecropin A. Thus, *in vivo,* Eater may efficiently target and phagocytose Gram-positive bacteria, but in order to recognize Gram-negative bacteria, AMP activity may be required to expose previously hidden Eater ligands. To assess the relative importance of Eater recognition and phagocytosis of Gram-positive pathogens, the group conducted *in vitro* binding assays and *in vivo* phagocytosis and survivals for three types of Gram-positive bacterial pathogens. Eater is important for the phagocytosis of *S. aureus* and *E. faecilis in vitro,* but is not required for phagocytosis of *Micrococcus luteus* (Chung and Kocks, 2011; Nehme et al., 2011)).

Another *Drosophila* hemocyte cell line, Kc167, does not express *eater* but is still able to efficiently phagocytose *M. luteus* but not *S. aureus* (Nehme et al., 2011). The importance of Eater in the host defense against Gram-positive bacteria was assessed in flies infected with three different Gram-positive bacteria: *S. aureus, E. faecalis,* and *Micrococcus luteus* (Nehme et al., 2011). *eater* null flies were susceptible to *S. aureus* and *E. faecalis* but showed little to no susceptibility after *M. luteus* infection. This finding is consistent with the phagocytic characteristics of Eater. Additionally, augmenting the host response by blood-cell specific activation of the Toll signaling pathway protected *eater* null flies against *E. faecilis* but not against *S. aureus*. Thus, in addition to the cellular immune response, the humoral response is effective against some Gram-positive bacteria (*E. faecilis*) but not others (*S. aureus*). The redundancy in

receptors and the overlapping effector mechanisms of the humoral and cellular immune responses are essential for the fly to mount an effective immune response. However, these responses and the interactions between them are unique to each pathogen the fly encounters, highlighting the complexity of the innate immune response in *Drosophila*.

Recently a bioinformatics study of immune related genes in the mosquito *Anopheles gambiae* identified *AgEater,* a gene that is translated into a plasma membrane bound receptor with 21 NIM repeats (Midega et al., 2013). This study was the first to identify and characterize the only two known NIM-repeat containing proteins in the mosquito, *AgNimB2* and *AgEater.* Surprisingly, injection of *AgEater* dsRNA into adult mosquitos, to specifically silence the expression of *AgEater,* did not affect phagocytosis of either *S. aureus* or *E. coli.* This finding stands in contrast to what is known about the function of *Drosophila* Eater, suggesting that AgEater is not required, or plays a redundant role, in bacterial phagocytosis in the mosquito.

To identify hemocyte-specific molecules, the Hultmark group generated a set of monoclonal antibodies against hemocytes (Kurucz et al., 2003). The research group used the antibodies to identify a plasmatocyte-specific EGF-domain containing transmembrane protein, Nimrod-C1 (NimC1) (Kurucz et al., 2007b). Specifically, two monoclonal antibodies, P1a and P1b, recognized different epitopes on NimC1 and immunofluorescence staining showed that both epitopes were found on the majority of larval hemocytes (with plasmatocyte morphology) but were absent on lamellocytes and crystal cells. The P1 antigen was immunoprecipitated and analyzed with MALDI-TOF mass spectrometry. The P1 target was identified as a 90-100 kDa single-pass transmembrane protein with ten NIM repeats, which the authors named Nimrod-C1.

Interestingly, the P1 antibodies did not recognize any antigens on S2 cells, indicating that *nimC1*is not expressed in this cell line, perhaps explaining why this receptor was not identified in the whole genome RNAi screens conducted by the Ezekowitz group. NimC1 localizes to the plasma membrane of larval hemoctyes. FACS analysis of the levels of P1 antigen on the surface of *nimC1* RNAi-silenced hemocytes led to decreased NimC1 protein on the surface of the cells. RNAi-mediated silencing of *nimC1* in larval hemocytes decreased *S. aureus* uptake to one-third of the controls but had no effect on *E. coli* phagocytosis. However, overexpression of NimC1 in S2 cells stimulated uptake of *S. aureus* and *E. coli* by 2.5-fold and 2-fold respectively. Thus, similar to Eater, NimC1 is important for *S. aureus* phagocytosis in plasmatocytes, and may play a redundant role for *E. coli* phagocytosis. Interestingly, NimC1 overexpression did not change the amount bacteria that bound to S2 cells, but did lead to increased uptake. Based on this result, it is unlikely that NimC1 directly bind to the microbe, but instead it may act a co-receptor, perhaps with Eater. Alternatively, NimC1 could be important for a later stage of the phagocytosis process, such as particle engulfment.

The importance of Eater and NimC1 for *E. coli* phagocytosis was recently assessed using an *in vivo* phagocytosis assay in *eater* or *nimC1* RNAi flies (Horn et al., 2014). QPCR analysis confirmed that hemocytes expressing the RNAi transgenes show reduced levels of *nimC1* or *eater* mRNA. The researchers counted the number of bacteria in adult hemocytes by injecting fluorescently-labeled *E. coli* and imaging individual hemocytes in the dissected dorsal vessel. Downregulation of *eater* and *nimC1* caused a modest but significant reduction in *E. coli* phagocytosis in adult hemocytes. In contrast, the Hultmark group's larval phagocytosis assay showed that *E. coli* phagocytosis was not

significantly affected when *nimC1* is downregulated via RNAi. One possible explanation for this discrepancy may be that NimC1 plays a more important role in *E. coli* phagocytosis at latter stages in development. The discrepancy between the function of NimC1 in larval hemocytes versus adult hemocytes could also be attributed to the nature of the experiments themselves. The larval phagocytosis assay was carried out *ex vivo* while the adult phagocytosis assay was done *in vivo.* It is possible that to facilitate phagocytosis of *E. coli,* NimC1 may need to interact with host factors that are only present *in vivo*. To more fully understand the function of NimC1, additional biochemical and genetic studies will be useful. In particular, it will be helpful to repeat the larval *E. coli* phagocytosis assay of *nimC1* RNAi by directly injecting the bacteria into the larvae and then quantifying the numbers of bacteria contained within the hemocytes. These conditions are more physiologically relevant than the *ex vivo* phagocytosis assay utilized Kurucz and colleagues and may help to reveal the function of NimC1 in the animal.

The final *Drosophila* Nimrod family member characterized as a phagocytic receptor is Draper, which is expressed in two types of phagocytes, glial cells and hemocytes (Freeman et al., 2003). During *Drosophila* neuronal development, selective pruning of axons is carried about by glia, cells that phagocytose apoptotic axons (Awasaki and Ito, 2004). *Draper* is the homolog of *ced-1*, a gene that encodes a receptor for apoptotic cells in *C. elegans* (Zhou et al., 2001). Nakanishi and group showed that Draper is an important receptor involved in the phagocytosis of apoptotic cells by glia and hemocytes (Manaka et al., 2004). The *C. elegans* homolog of Draper, CED-1, acts upstream CED-6, an adaptor protein that serves as a molecular scaffold for signaling complexes at the phagocytic cup (Zhou et al., 2001). CED-1 contains an intracellular

NPxY motif that is a binding site for proteins containing a phosphotyrosine binding (PTB) domain, an YxxL motif, a domain that is a potential binding site for proteins containing Src-homology-2 domains (SH2). In *C. elegans,* the phosphotyrosine binding domain (PTB) adaptor protein, CED-6 binds to the CED-1 NPxY motif to promote phagocytosis of cell corpses (Liu and Hengartner, 1998; Su et al., 2002) *Drosophila* Ced-6 is an adaptor protein with an SH2 domain and a Pleckstrin-homology (PH) domain. The SH2 domain of Ced-6 binds to a phosphorylated tyrosine in the NPxY motif in the intracellular region of activated Draper, and the PDZ domain recruits downstream factors important for apoptotic cell clearance and phagocytosis of bacteria (Awasaki et al., 2006; Fujita, 2012; MacDonald et al., 2006; Ziegenfuss et al., 2008).

Franc and group confirmed the role for Draper and Ced-6 in phagocytosis of apoptotic cells and further examined the possibility of a role for Draper in phagocytosis of bacteria (Cuttell et al., 2008). The heat-killed bioparticles used by the authors were conjugated to the pH-sensitive dye pHrodo, which fluoresces at low pH (~4.5). After being engulfed by the cell, pHrodo-labeled particles will only fluoresce in the acidic environment of the phagolysosome. *draper* RNAi treated S2 cells showed significantly less fluorescence of pHrodo-labeled bacteria than untreated cells, with only 35% and 40% of the cells showing fluorescence of pHrodo-labeled *E. coli* and *S. aureus,* respectively. Additionally, *draper* and *ced-6* mutant flies injected with pHrodo-*S. aureus* and *E. coli* showed reduced fluorescence in their dorsal vessels. The lack of fluorescence could be indicative of a decreased uptake and/or impaired maturation of the phagosome.

The apoptotic cell ligand for Draper has been the subject of considerable research by the Nakanishi group. The initial paper that characterized a role for Draper in

apoptosis determined that the receptor does not recognize the common "eat me" signal, phosphatidylserine (PS) (Manaka et al., 2004). The same group later determined that the endosomal protein Pretaporter is exposed on the surface of apoptotic cells and serves as a ligand for Draper. Additionally, the group extended their genetic studies and found that Rho-GTPases *Rac1* and *Rac2* are involved in the Draper/Ced-6 pathway to engulf apoptotic cells. Finally, they re-examined the possibility Draper binds PS using biochemical and genetic techniques. They first tested binding of Draper to PS using exogenously expressed Draper proteins and an ELISA-like activity assay and found that full length Draper efficiently binds to PS while truncated Draper proteins did not (Tung et al., 2013). Finally, they performed an *in vivo* assay in embryos comparing apoptotic cell phagocytosis in *draper* null flies expressing either wildtype Draper or a truncated Draper protein, Draper-ΔEN (A construct that generated a Draper protein missing the N-terminal EMI and NIM domains). Expression of wildtype Draper protein led to increased uptake of apoptotic cells. In contrast Draper-ΔEN did not lead to an increase in phagocytosis, and shared a phenotype similar to control *draper* null flies. Thus, Draper was unable to function as a phagocytic receptor when the region containing the EMI and NIM domains was deleted, indicating that N-terminal domain region of the protein is critical to Draper's role as a receptor for apoptotic cells.

The *S. aureus* ligand for Draper was determined in a well-designed genetic study by Shiratsuchi and group (Hashimoto et al., 2009). The group utilized *S. aureus* mutant strains with defects in the structure of the cell wall to screen for bacterial ligands whose loss impaired the recognition and uptake of the bacteria by larval hemocytes. About half of the tested strains were 30-60% less efficiently phagocytosed, and many of the genes

mutated in these strains were involved in the synthesis of teichoic acid. One of the strains less efficiently phagocytosed had a mutation in the gene *ltaS,* which encodes an enzyme necessary for the synthesis of polyglycerolphosphate of lipoteichoic acid (LTA). This phenotype was recapitulated in adult flies: FITC-labeled *ΔltaS* bacteria were phagocytosed less efficiently than the parental strain. The *ΔltaS* bacteria were also more virulent than the parent strain; causing higher bacteria loads 15-18 hours after injection into adult flies. The survival and bacteria load phenotypes were reversed by expression of wildtype *ltaS* in the *ΔltaS* background. The humoral immune response of flies infected with *ΔltaS S. aureus* was unaltered, indicating that the increased pathogenicity associated with the *ΔltaS* mutation could be attributed, in part, to decreased bacterial clearance by phagocytic cells. This finding highlights the vital role of phagocytes in the immune response to *S. aureus*.

Importantly, the authors determined that Draper was a receptor for LTA on the surface of *Drosophila* hemocytes. To identify the hemocyte receptor for LTA, the authors tested all characterized Nimrod-superfamily receptors, Eater, NimC1 and Draper. Hemocytes lacking Eater phagocytosed the parental *S. aureus* strain less than efficiently controls, a finding that was consistent with work by the Stuart group (Kocks et al., 2005). However, *Eater* null hemocytes also phagocytosed *ΔltaS S. aureus* less efficiently, indicating that Eater is not required for *ltaS*-dependent phagocytosis of *S. aureus.* Additionally, NimC1 is not required for *ltaS*-dependent phagocytosis of *S. aureus,* as phagocytosis assays using l(2)mbn cells, a cell line derived from larval hemocytes, showed similar results. However, larval and adult hemocytes from *draper* null mutants equally phagocytosed parental and *ΔltaS S. aureus* indicating that Draper is responsible

for *ltaS*-dependent phagocytosis. *Draper* null hemocytes also showed decreased phagocytosis of *Bacillus subtilis,* another Gram-positive bacteria that expresses LTA containing polyglycerolphosphate. Thus Draper is important for the recognition of this component of the cell wall for more than one Gram-positive bacterial species (Hashimoto et al., 2009). Finally, Franc and group previously demonstrated that Draper is important for *E. coli* phagocytosis in adult flies (Cuttell et al., 2008). Later, the Shiratsuchi group confirmed this finding using larval hemocytes from *draper* null mutants (Hashimoto et al., 2009). Together this data demonstrates the Draper is a receptor for multiple ligands. It recognizes LTA on the surface of Gram-positive bacteria, phosphatidylserine and Pretaporter on the surface of apoptotic cells, and undetermined ligands on the surface of Gram-negative bacteria.

Draper is a multifunctional receptor with wide-ranging ligand specificity, an important feature for a receptor found on the surface of invertebrate phagocytes. Ligand-dependent activation of Draper may initiate specific downstream signaling events that instruct the cell as to what type of particle is to be phagocytosed. Draper-mediated phagocytosis of apoptotic corpses requires phosphorylation of the immune receptor tyrosine-based activation motif (ITAM) in the Draper intracellular domain. Shark, the *Drosophila* counterpart of Syk and Zap70, is Src-family kinase that mediates Draper ITAM phosphorylation in glial cells, promoting apoptotic cell phagocytosis (Fujita, 2012; Ziegenfuss et al., 2008). The relative importance of Draper ITAM phosphorylation has not been examined with respect to phagocytosis of bacteria. The adaptor protein Ced-6 interacts with Draper to mediate uptake of bacteria and this interaction is most likely dependent on the recognition of a phosphorylated tyrosine in Draper's intracellular

region. It is unknown, however, if Shark, or some other *Drosophila* tyrosine kinase, phosphorylates the Draper ITAM in response to bacteria. Thus activation of Draper and the assembly of downstream signaling cascades may be ligand-dependent, a possibility that might add yet another layer of complexity to the function of Draper.

Peptidoglycan-recognition receptors important for phagocytosis:

The peptidoglycan recognition proteins (PGRPs) are important microbial receptors that were first identified in the hemolymph of silkworms, *Bombyx mori* (Yoshida et al., 1996). PGRPs were also found and in *Trichoplusia ni*, moth, larvae immune challenged with the Gram-positive bacteria *Enterobacter cloacae* (Kang et al., 1998). The report by Yoshida *et al.* demonstrated that PGRPs bind to peptidoglycan (PGN), a complex polymer consisting of sugars and amino acids that is restricted to the cell wall of Gram-positive and Gram-negative bacteria (Yoshida et al., 1996). Peptidoglycan is made up of alternating *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc) residues that are cross-linked to each other by short peptide bridges of three to five amino acids. PGN of most Gram-positive bacteria contains a lysine residue as the third amino acid in the peptide chain and is known as Lys-PGN. Gram-positive bacilli and Gram-negative bacteria have meso-diaminopimelic acid as the third amino acid (DAP-type PGN). Another feature that is unique to DAP-type PGN is the presence of a monomer, known as tracheal cytotoxin (TCT), on the terminal PGN unit. Finally, Gram-positive and Gram-negative bacteria PGN differ in their localization within the cell wall. DAP-type PGN forms a single layer that is hidden in the periplasmic space beneath the outer membrane and lipopolysaccharide (LPS) layer of the cell wall of Gram-negative bacteria. In Gram-positive bacteria, PGN is highly

abundant and can account for half of the mass of the cell wall. Gram-positive bacteria PGN form a multilayer structure that is exposed on the surface (Royet and Dziarski, 2007).

The Steiner group performed an elegant study to characterize the structure and relatedness of insect and mammalian PGRPs. They cloned PGRP from moth, mouse, and human samples and found that transcripts corresponding to PGRP were highly expressed in organs of the immune system. Comparison of the predicted amino acid sequences of PGRPs revealed that murine and human PGRPs share 43% sequence identity with *T. ni* PGRP. Additionally, mammalian PGRPs function in a manner analogous to insect PGRPs, as demonstrated by an experiment where recombinant murine PGRP bound to PGN in a manner similar to *T. ni* PGRP. Further examination of the structure of *T. ni* PGRP revealed that the protein shared 28% identity and 50% similarity with bacteriophage T7 lysosome, a zinc-dependent *N*-acetylmuramoyl-L-alanine amidase (Kang et al., 1998). *N*-acetylmuramoyl-L-alanine amidases cleave peptidoglycan at the lactylaminde bond, removing the peptidic bridge from the sugar backbone. Interestingly, recombinant *T. ni* PGRP showed no amidase activity on *E. coli* cell walls. This observed lack of amidase activity could be explained by the fact that *T. ni* PGRP lacks the zinc-binding residues present in the phage enzyme and suggests the primary function of the *T. ni* PGRP was recognition and binding of PGN.

There are 13 PGRP genes in *Drosophila* and studies in the fruit fly model system have provided the most comprehensive data on PGRPs. *Drosophila* PGRPs recognize microbial ligands upstream of the Toll and IMD signaling pathways, the major signaling cascades regulating the humoral innate immune response. Briefly, the Toll signaling

pathway is activated after infection with fungi, Gram-positive bacteria or Drosophila X virus, while the IMD pathway is activated by Gram-negative bacteria. Activation of the Toll and IMD pathways leads to the production of systemic antimicrobial peptides and other immune responsive effectors. (The Toll and IMD pathways will be discussed in greater detail later in this review).

Six *Drosophila* PGRP genes code for long (L) forms, four of which are transmembrane proteins localized at the plasma membrane. The remaining seven PGRP genes are short (S) forms that are predicted to be secreted (Werner et al., 2000). *Drosophila* PGRPs can also be divided based on their recognition and/or catalytic properties. Members of the non-catalytic group (PGRP-SA, SD, LA, LC, LD, LE and LF) serve as microbial sensors and PRRs. These PGRPs lack the critical cysteine residue in the enzymatic pocket of the PGRP domain and are unable to degrade PGN (Mellroth et al., 2003). The second group, catalytic PGRPs have either been experimentally verified (PGRP-SC1, LB, SB1) or predicted (SC2 and SB2) to possess amidase activity needed to degrade PGN (Bischoff et al., 2006; Mellroth et al., 2003; Mellroth and Steiner, 2006; Zaidman-Remy et al., 2006; Zaidman-Remy et al., 2011).

Finally, recognition of PGN plays a critical role in host defense in *Drosophila.* Evidence supporting the importance of recognition of PGN by phagocytes recently came from adult and larval phagocytosis studies using an *S. aureus* strain with temperature sensitive UDP-*N*-acetylenolpyruvylglucosamine reductase (*murB*). This mutant *S. aureus* strain produces reduced levels of peptidoglycan at non-permissive temperatures. *Drosophila* hemocytes phagocytosed *MurB* bacteria 50% less efficiently than wild type *S.*

aureus, and this phenotype could be rescued by complementation of the mutant strain with the wild type gene (Shiratsuchi et al., 2012).

An *in vivo* study using RNAi to deplete *PGRP-SC1/2* revealed that PGRP-SC1 and SC2 modulate the innate immune response by scavenging PGN, effectively controlling inflammation and damage to host tissues (Bischoff et al., 2006). RNAi of *PGRP-SC1/2* in flies led to over-activation of the IMD pathway following infection with Gram-negative bacteria. Interestingly, activation of the Toll pathway was unaffected in *PGRP-SC1/2 RNAi* flies after Gram-positive bacterial challenge, indicating that PGRP-SC1 and SC2 act upstream of the IMD signaling pathway. Coincidently, this finding contradicted an earlier study by the Wu laboratory, which found that PGRP-SC1a is a receptor for the Gram-positive bacteria, *S. aureus*, and not for Gram-negative bacteria (Garver et al., 2006). Using an adult, *in vivo* phagocytosis assay, the Wu group screened a collection of ethylmethane sulfonate (EMS) mutated flies (Koundakjian et al., 2004). One mutant, *picky eater* (*picky*), was defective for *S. aureus* phagocytosis (25% of tested flies took up the fluorescein-labeled particles). However *picky* flies were able to efficiently phagocytose *E. coli* and *Saccharomyces cerevisiae* zymosan particles as well as live, GFP expressing *Bacillus subtilis* (a Gram-positive bacteria possessing DAP-type PGN). The *picky* mutant was also impaired for survival after *S. aureus* infection. The *picky* mutation mapped to the catalytic PGRP gene, *PGRP-SC1a*. Both the impaired recognition of *S. aureus* and survival of *picky* mutants were rescued by transgenic expression of *PGRP-SC1a.* The catalytic activity of PGRP-SC1a was required for phagocytosis and clearance of *S. aureus* since a non-catalytic PGRP-SC1a (in which the critical cysteine residue is replaced by a serine) was not sufficient to rescue phagocytosis

or survival after *S. aureus* infection. This data provides strong evidence for the role of PGRP-SC1a as a PRR in the fruit fly. Discrepancies in the findings presented in the Bischoff *et al.* and the Garver *et al.* papers may have arisen from the way PGRP-SC1a expression was altered in each study. In particular, the RNAi transgene utilized in the Bischoff *et al.* paper targeted not only PGRP-SC1a but also PGRP-SC1b and PGRP-SC2, while the *picky* mutation specifically affected the expression of PGRP-SC1a. Thus the strong over-activation of the IMD pathway that was observed by this group may have been attributed to additional loss-of-function of PGRP-SC1b and/or PGRP-SC2.

Another PGRP that acts as a PRR in *Drosophila* is PGRP-SA. A screen to identify mutations that impair the production of the Toll pathway responsive antimicrobial peptide, Drosomycin, following Gram-positive infection identified the mutation *semmelweis* (*seml*) (Michel et al., 2001b). The *seml* mutation is caused by an amino acid change in the PGRP domain, cysteine 80 to tyrosine 80, and this change effectively inactivated the PGRP-SA gene. After infection with Gram-positive bacteria, but not fungi, *seml* flies are unable to produce Drosomycin due to impaired Toll activation (Michel et al., 2001b). In addition to its role in activating the Toll pathway, PGRP-SA may also be important for phagocytosis of Gram-positive bacteria. Specifically, Garver *et al*. tested *seml* mutants using the adult *in vivo* phagocytosis assay and found that 94% of *seml* mutants efficiently phagocytosed *E. coli,* while only 25% were able to phagocytose *S. aureus* (Garver et al., 2006). In contrast, a separate study looking at *in vivo* phagocytosis of *S. aureus* in *seml* mutants failed to observe an effect on *S. aureus* phagocytosis in adult flies (Nehme et al., 2011). This experiment however, only tested phagocytosis in 10 wild type and 12 *seml* flies. The discrepancy observed

between the two papers could be addressed by additional experiments. In particular, the experiment should be performed in triplicate in order to draw reliable conclusions about the phagocytosis phenotype of the *seml* mutant. For that reason the Wu laboratory conducted additional, independent, *in vivo S. aureus* phagocytosis experiments using *seml* mutants. They found that *seml* flies did indeed show defective *S. aureus* phagocytosis (unpublished A. Nazario-Toole) as previously reported.

This Ezekowitz group established that *Drosophila* S2 cells are a valid *in vitro* system for studying phagocytosis and using this system, they found that dSR-CI is a receptor for bacteria (Ramet et al., 2001). This group carried out another RNAi screen in S2 cells and identified PGRP-LC as important for phagocytosis of Gram-negative (*E. coli*) but not Gram-positive (*S. aureus*) bacteria (Ramet et al., 2002). PGRP-LC is a non-catalytic, membrane-bound PGRP in *Drosophila.* Decreased expression of *PGRP-LC* led to a 30% reduction in *E. coli* phagocytosis and also affected binding of the bacteria to the cell surface. This modest decrease in phagocytosis observed in S2 cells was likely due to the fact that other receptors, such as Eater or dSR-CI, participate in recognition and uptake of Gram-negative bacteria. The group was also interested in determining if PGRP-LC functions during the humoral immune response and, using oligonucleotide microarrays, they measured the induction of genes after 6 hours of exposure to *E. coli.* RNAi of *PGRP-LC* in S2 cells dramatically reduced the expression of genes regulated by the IMD pathway, such as the antimicrobial peptide *Attacin.* To test the role of PGRP-LC *in vivo,* the group generated *PGRP-LC* mutants: *Δ5* is a null allele and *N18* is a hypomorphic allele. Both *Δ5* and *N18* flies were more susceptible to *E. coli* and this susceptibility was accompanied by a reduced expression of IMD-regulated AMPs (Ramet

et al., 2002). A paper published simultaneously by the Anderson group identified an EMS mutant, *ird7*, that was defective in the induction of AMPs by the IMD pathway (Choe et al., 2002). The defects observed in *ird7* mutants were similar to those observed in *imd* mutants. The *ird7* mutation mapped to the genomic locus containing *PGRP-LC* and *PGRP-LA*. Sequence analysis of *ird7* mutants showed that the mutation would disrupt the function of *PGRP-LC*. Yet another group, the Royet group, published a paper in the same month that identified a P transposon insertion mutant with compromised induction of IMD responsive genes after Gram-negative infection (Gottar et al., 2002). This mutant line, *PGRP-LC*^{7454}, had a transposable element inserted in the first exon of the *PGRP-LC* gene, and these flies were more susceptible to Gram-negative infections but were similar to wild type flies after infection with Gram-positive or fungal pathogens. Together the work of these groups clearly established that PGRP-LC is the major receptor upstream of the IMD pathway *in vivo* and *in vitro.*

Although the data supporting a role for PGRP-LC during the humoral response to Gram-negative bacteria was in agreement, the importance PGRP-LC as a phagocytic receptor was less clear. The Ezekowitz group showed that PGRP-LC led to a modest decrease in *E. coli* phagocytosis *in vitro,* they did not explore the of role of PGRP-LC as a phagocytic receptor *in vivo* (Ramet et al., 2002). Two separate groups reported that blood cells from the *ird7* mutant are able to efficiently phagocytose Gram-positive and Gram-negative bacteria (Choe et al., 2002; Garver et al., 2006). This discrepancy may be attributed to the fact that the Ezekowitz group studied phagocytosis in S2 cells after all splice forms of *PGRP-LC* were silenced. Three isoforms are produced from *PGRP-LC, -LCa, -LCx*, and *–LCy* (Werner et al., 2000). The three transcribed proteins all share the

same intracellular, signaling, domains but have unique extracellular domains. PGRP-LCx recognizes PGN purified from *E. coli* as well as the TCT fragment of PGN. In contrast PGRP-LCa specifically recognizes TCT (Kaneko et al., 2004). The *ird7* mutation caused an amino acid change in the PGRP domain of PGRP-LCx while RNAi treatment would cause decreased expression of all isoforms. It is possible that the unaltered expression of *PGRP-LCa* in the *ird7* mutant is sufficient to allow for PGRP-LC mediated uptake of *E. coli in vivo.* Additionally, other *E. coli* pattern recognition receptors may participate in phagocytosis *in vivo.*

Recent work by the Fauvarque group may help support a role for PGRP-LC in the phagocytosis of Gram-negative bacteria (Bergeret et al., 2008; Perrin et al., 2015). Based on evolutionary and functional conservation of nonaspanins (TM9 protein family), proteins characterized by the presence of a large extracellular N-terminal domain and nine transmembrane domains, the Fauvarque group initiated a genetic and phenotypic analysis to characterize the function of TM9 proteins in *Drosophila.* There are three *TM9* genes in *Drosophila*, and the group created a mutant with a deletion of one *TM9* gene, *TM9SF4* (Bergeret et al., 2008). The *TM9SF4* mutant was more susceptible to pathogenic Gram-negative infection (*Klebsiella pneumoniae* and *Enterobacter cloacae*) but showed normal resistance to non-pathogenic Gram-negative bacteria (*E. coli*) and Gram-positive bacteria. Additionally, the Toll and IMD responsive genes were not affected in *TM9SF4* mutants. However, *TM9SF4* mutant adults showed less efficient phagocytosis of GFP-labeled *K. pneumoniae*, with mutant flies showing higher levels of bacteria in hemolymph bled from infected flies. Using an *ex vivo* phagocytosis assay, the authors also found that *TM9SF4* mutant larval hemocytes phagocytosed *E. coli* two times less

efficiently than wild type hemocytes. The group explored the possibility that PGRP-LC and TM9SF4 interact *in vivo* due to the fact that *TM9SF4* mutants and *PGRP-LC* RNAi of S2 cells both exhibit specific defects in the phagocytosis of Gram-negative bacteria (Perrin et al., 2015). In S2 cells, GFP-tagged TM9SF4 and V5 epitope-tagged PGRP-LC co-immunoprecipiate*,* indicating that the proteins do indeed interact. Furthermore, in the fat body, the functional equivalent of the mammalian liver, GFP-tagged TM9SF4 and FLAG-tagged PGRP-LC co-localized at the plasma membrane. Importantly, in S2 cells, TM9SF4 is required for PGRP-LC localization to the plasma membrane and the observed reduced phagocytosis of Gram-negative bacteria in *TM9SF4* null flies may be due to a loss of PGRP-LC at the plasma membrane.

To fully characterize the importance of PGRP-LC during the cellular immune response to Gram-negative bacteria in *Drosophila,* it may be necessary to carry out the adult and larval *in vivo* phagocytosis assays in *TM9SF4* mutants, *PGRP-LC*^{7454} transposon insertion mutants, *PGRP-LC* null mutants, or in flies with *PGRP-LC* silenced specifically in hemocytes with RNAi. Such a comprehensive study would serve to clarify the relative contributions of PGRP-LC alternatively spliced isoforms during the cellular immune response in the fly. Finally, a study in adult *Anopheles gambiae* mosquitos showed that down-regulation of *PGRP-LC* by injecting dsRNA specifically affected phagocytosis of *E. coli,* providing additional evidence in another insect that PGRP-LC is a phagocytic receptor for Gram-negative bacteria (Moita et al., 2005).

Integrin αPS3/βv:

The Nakanishi group, the group that found Draper is a phagocytic receptor, characterized a role for the integrin heterodimer, αPS3 and βv, in the phagocytosis of *S.*

aureus and apoptotic cells (Nagaosa et al., 2011; Nonaka et al., 2013; Shiratsuchi et al., 2012). Using a procedure described in Kurucz *et al.,* this group raised monoclonal antibodies against *Drosophila* hemocytes by immunizing mice with larval hemocytes (Nagaosa et al., 2011). The group then added each antibody to culture dishes containing the larval phagocytic cell line, l(2)mbn, co-incubated with chemically killed S2 cells and looked for effects on phagocytosis of S2 cells by the l(2)mbn cell line. Treatment with an antibody that recognized an extracellular Perlecan-like protein, Trol, led to decreased uptake of the dead S2 cells. The extracellular region of the Trol protein has 3 RGD domains, a motif this is found in the ligand for some groups of integrins. The binding of integrin to RGD ligands induces the phosphorylation of the tyrosine kinase, Focal adhesion kinase (FAK) (Shattil et al., 2010). Treatment of l(2)mbn cells treated with recombinant Trol protein resulted in a 1.5 fold increase in the levels of phosphorylated FAK, indicating that Trol binds to integrin on the surface of l(2)mbn cells.

Integrin functions as a heterodimer of two transmembrane subunits, α and β integrin. In the *Drosophila* genome, 5 genes code for the α subunit and 2 genes code for the β subunit (Brown et al., 2000). The Nakanishi group initially focused on the examining a role for the two β subunits. To do so, they examined phagocytosis of dead S2 cells by Croquemort-positive hemocytes derived from mutant flies lacking either integrin β subunit gene. Cells derived from *Integrin bena nu* (*Itgbn*) mutant embryos displayed apoptotic cell phagocytosis defects. Antibody staining confirmed that the βν protein is found on the surface of embryonic hemocytes. To determine the relationship of integrin βν with the known apoptotic cell phagocytic receptor, Draper, the authors analyzed phagocytosis of S2 dead cells by embryonic hemocytes derived from *Intbn* and

drpr single and double mutants. Simultaneous loss of both receptors decreased phagocytosis of dead S2 cells to half that of single mutants, indicating that Draper and integrin βν are independent receptors for apoptotic cells (Nagaosa et al., 2011).

The group then expanded their studies to examine a role for integrin βν in the phagocytosis of *S. aureus.* Similar to what was observed with apoptotic cells, adult *Intbn* and *drpr* double mutant flies phagocytosed *S. aureus* less efficiently than single mutant flies, indicating that Draper and integrin βν also act independently as receptors during *S. aureus* phagocytosis. Importantly, integrin βν-deficient adult and larval hemocytes phagocytose *S. aureus* less efficiently than control flies, but are able to phagocytose *E. coli* and the DAP-type PGN-containing Gram-positive bacteria *B. subtilis* with the same efficiency as wild type flies. Adult flies lacking the integrin βν subunit were more susceptible to septic *S. aureus* infection. Additionally, these flies carried a higher bacterial load indicating that integrin βν mediated phagocytosis of *S. aureus* limits bacterial growth within the fly. Thus, integrin βν is a receptor for *S. aureus* (and possible for other Gram-positive bacteria) found on the surface of hemocytes that plays a critical role in the host cellular immune response.

The Nakanishi group then sought to determine which component of the cell wall of *S. aureus* is recognized by integrin βν. To do so, they carried out binding assays utilizing an *S. aureus* strain carrying a mutated *murB* gene. *murB* encodes UDP-*N*-acetylenolpyruvylglucosamine reductase, a key enzyme in bacterial peptidoglycan synthesis, and *murB* mutant *S. aureus* contain significantly less peptidoglycan polymers in their cell wall than their wild type counterparts. The *murB* mutant *S. aureus* strain is a less efficient target for phagocytosis by normal hemocytes than the parental *S. aureus*

strain. To examine the binding of integrin βν to *S. aureus* strains, the authors incubated either *murB* mutant or parental *S. aureus* bacteria with a GST-fused recombinant integrin βν protein. Cell lysates were examined with anti-GST antibodies using western blotting to determine which strain of bacteria more efficiently bound to integrin βν. The wildtype *S. aureus* bound to integrin βν more efficiently than the *murB* mutant strain. Finally, GST-fused integrin βν bound to culture dishes was able to adhere to a solid phase preparation of *S. aureus* peptidoglycan in a dose dependent manner, but this was not the case with GST alone. Together, these binding assays show that integrin βν binds to *S. aureus* peptidoglycan and this physical association may be critical for integrin βν's role as a phagocytic receptor.

Drosophila integrin functions as a heterodimer of β and α subunits. The identity of the α subunit that forms a complex with integrin βν was determined in a recent paper from the Nakanishi lab (Nonaka et al., 2013). Nonaka and colleagues utilized RNA-interference to silence the expression of each of the five *Drosophila* α subunit genes specifically in hemocytes. They assessed ability of the α integrin-depleted larval hemocytes to phagocytose apoptotic cells and identified one subunit, αPS3, whose loss impaired phagocytosis. αPS3 integrin protein is coded by *scab* (*scb*). *scb* deficiency flies (mutant flies with a deletion in the chromosome region that includes *scb*) and flies with a P-element insertion that disrupts the coding region of *scb,* both show a reduction in the level of apoptotic cell clearance. Forced expression of wildtype αPS3 in the *scb* deficiency mutant was sufficient to restore the phagocytosis of apoptotic cells, indicating that αPS3 is the *Drosophila* α integrin subunit required for the recognition and uptake of dead cells *in vivo*. To assess a functional interaction between βν and αPS3, the authors

generated flies with hemocyte-specific RNAi- mediated silencing of the genes coding for βv (*Itgbn*), αPS3 (*scb*), or both βv and αPS3. In all three types of embryos, only about 20% of the hemocytes were able to phagocytose apoptotic cells. Because phagocytosis of apoptotic cells occurred almost equally in the three fly lines, βv and αPS3 function in the same pathway during phagocytosis in embryonic hemocytes. A similar approach was taken to assess a functional interaction between αPS3 and βv during phagocytosis of *S. aureus*. Embryonic hemocytes from flies with *Itgbn* and *scb* silenced together, or alone, showed equal levels of *S. aureus* phagocytosis. These results indicate that αPS3 and βv form a heterodimer that serves as a phagocytic receptor for *S. aureus.* Importantly, the physical association of αPS3 and βv was confirmed through immunoprecipitation and western blotting of l(2)mbn cell lysates. In conjunction, the genetic and biochemical analyses carried out by the Nakanishi group clearly establish that the αPS3/βv integrin heterodimer is a phagocytic receptor for apoptotic cells and *S. aureus* in *Drosophila.*

Down-syndrome adhesion molecule 1 (Dscam 1):

The pattern recognition receptors of innate immunity are effective in recognizing a wide array of pathogen associated molecular patterns. However, innate immune responses are constrained to structures that are common to pathogens and conserved during evolution. In contrast, receptors of the adaptive immune response are able to recognize an almost infinite diversity of antigens through somatic rearrangements of genes. Members of the Immunoglobulin superfamily (IgSF) of proteins, such as antibodies and the antigen receptors found on the surface of B and T lymphocytes, are an essential part of mammalian adaptive immune responses.

One IgSF member is *Drosophila Down-syndrome adhesion molecule 1* (*Dscam1*). The *Drosophila* genome contains four Dscam-like genes and the most extensively characterized of these is *Dscam1* (Armitage et al., 2012; Vogel et al., 2003). The *Dscam1* gene is arranged into clusters of variable exons (exons 4, 6, 9 and 17) that are flanked by constant exons. Mutually exclusive alternative splicing of the variable exons generates a large protein isoform repertoire that has the potential to recognize and bind diverse ligands (Schmucker et al., 2000). Dscam1 is critical for nervous system development and is essential for axon guidance and the formation of neural connections in *Drosophila* (Wojtowicz et al., 2004; Zhan et al., 2004).

To explore a potential role for the hypervariable Dscam1 receptor in the immune response to bacteria, the Schmucker group conducted a functional analysis of *Dscam1* expression in immune competent tissues of *Drosophila* (Watson et al., 2005). In situ hybridization of larval tissue revealed that *Dscam1* is expressed in neural tissue, hemocytes and fat body tissue. cDNAs derived from all three tissues were hybridized to microarrays containing 50-mer oligos for all alternatively spliced exons. Based on the number of alternatively spliced exons detected, an estimated 18,000 Dscam1 receptor isoforms are expressed in hemocytes and the fat body. The Dscam1 protein is expressed in immune tissues; antibodies against the common Dscam cytoplasmic region recognized Dscam in S2 cells, larval hemocytes, and larval fat bodies. Western blots using the anti-Dscam1 antibody revealed the presence of a soluble Dscam1 protein in S2 cell-conditioned medium and larval hemolymph. Secreted Dscam1 proteins could act as opsonins or receptors that recognize microbes present in the hemolymph of the fly.

GFP-positive hemocytes were purified from wild type larvae and from *Dscam* mutant larvae (with a transallelic combination of a hypomorphic and an amorphic *Dscam1*) and the amount of fluorescently-labeled *E. coli* phagocytosed by these hemocytes was determined using flow cytometry. Fifty-five percent of mutant hemocytes phagocytosed the bacteria while 90% of wild type hemocytes took up the bacteria. Silencing *Dscam1* expression using a hemocyte-specific promoter led to a 60% reduction in the number of hemocytes that phagocytosed *E. coli.* Additionally, treating S2 cells with anti-Dscam1 antibody (to block Dscam1 function by binding to the extracellular domain) also reduced the number of cells that could phagocytose *E. coli.* Both loss of Dscam1 expression and blocking Dscam1 function with antibodies caused significant phagocytosis defects, indicating that Dscam1 functions as a receptor for *E. coli.* To determine if Dscam1 directly binds to *E. coli,* the authors carried out binding assays that tested the binding of certain Dscam1 isoforms to live DH5α *E. coli.* Two Dscam1 isoforms, one containing all of the extracellular domain and another that contained only the N-terminal Ig-like domain and the first Fibronectin III domain were able to bind to the bacteria. However, another Dscam1 isoform containing the complete extracellular domain was unable to bind to *E. coli.* The distinct binding properties of tested Dscam1 isoforms hinted at the possibility that Dscam1 isoforms bind distinct microbial ligands, thus increasing the number of possible ligands recognized this receptor.

Data from the mosquito *Anopheles gambiae* clearly showed that Dscam1 isoforms show specificity for different microbes (Dong et al., 2006). The immune competent mosquito cell line, Sua5B, was challenged with Gram-positive bacteria, Gram-negative bacteria and the malaria parasite *Plasmodium berghei.* Quantitative RT-PCR analysis of

transcripts from challenged cells revealed rapid and robust changes in *AgDscam* exon usage. Each microbe induced distinctive splice isoform repertoires that would result in the production of AgDscam molecules with diverse binding properties. Importantly, splice isoforms elicited after a specific immune challenge showed higher binding affinity to the specific microorganism used during the challenge. This result suggests that alternative splicing of *AgDscam* plays a role in the mosquito's immune receptor diversity and specificity. The occurrence of pathogen induced *AgDscam* alternative splicing was also observed *in vivo.* Bacterial challenge of adult mosquitos triggered pathogen-specific alternative splicing of *AgDscam,* but it was unclear how hemocyte or fat body tissue splice repertoires differed in response to infection. Depletion of *AgDscam* via injection of double stranded RNA (dsRNA) targeting a non-alternatively spliced exon resulted in a 50% reduction in the levels of AgDscam protein in adults and *AgDscam dsRNA*-treated mosquitos were significantly more susceptible to *S. aureus* and *E. coli* infection than control flies treated with *GFP* dsRNA.

The Schmucker group found that *Drosophila* Dscam1 mediates phagocytosis of *E. coli* (Watson et al., 2005). As a follow-up to this finding, the Dimopoulos group assessed bacterial phagocytosis by *A. gambiae* immune competent Sua5B cells treated with *GFP dsRNA* or *AgDscam dsRNA* (Dong et al., 2006). Silencing *AgDscam* resulted in a 50% reduction in phagocytosis of both *E. coli* and *S. aureus* indicating that Dscam is an evolutionarily conserved phagocytic receptor. Studies of the immune function of the Dscam1 homolog in the crayfish, *Pacifastacus leniusculus,* also showed that bacterial infection induced the alternative splicing of isoforms with specific affinity to the bacteria used to infect the animal. Furthermore, as with the fruit fly and the mosquito, crayfish

Dscam was shown to mediate bacteria clearance and phagocytosis (Watthanasurorot et al., 2011). Hence, Dscam1 is a phagocytic receptor for bacteria and this function is conserved in invertebrates. It will be interesting to determine if Dscam1 pathogen-specific isoforms persist in the animal after an infection is cleared and if they serve as to prime the immune response to subsequent infections. Finally, if cells that produce alternatively spliced forms of Dscam in response to infection are able to persist and respond quickly to secondary infections with the same pathogen, it may be an example of convergent evolution of the immune system.

Opsonins in *Drosophila* phagocytosis:

Opsonization is the process by which soluble host molecules bind to and alter the surface of a pathogen or particle so that it can be ingested more efficiently by phagocytes. In mammals, antibody and complement factors act as opsonins. Insect TEPs (thioester-containing proteins) share sequence similarities with the vertebrate complement factors C3/C4/C4 and the α2-macroglobulin family of serine proteases. In vertebrate immunity, activated complement proteins, such as iC3b, form covalent bonds with molecules on the surfaces of pathogens or altered self. Complement attachment to the surface of target particles marks these cells for opsonization.

In *Drosophila* the TEP family is made up of six genes, *TEPI – TEPVI,* of which one, *TEPV,* does not seem to be expressed (Lagueux et al., 2000). All genes of this family possess a signal peptide, indicating that they are secreted proteins. TEPI – TEPIV are most closely related to complement factors, as they share a common CGEQ amino acid motif that is critical to the formation of thioester-bonds with target surfaces. *TEPVI* is also known as *macroglobulin complement related* (*Mcr*), and the Mcr protein differs

from the other TEPs in that it lacks the critical cysteine residue in the thioester-binding site (Stroschein-Stevenson et al., 2006). Phylogenetic analysis reveals that TEP proteins are found in nematodes, insects, mollusks, fish, birds, and mammals (Nonaka, 2000). A population genetic analysis of TEPI - TEPIV proteins in *Drosophila* showed that *TEPI* is under positive selection and is one of the most rapidly evolving genes in the *Drosophila* genome. Thus, it is possible that *TEPI* is evolving to adapt to new pathogens encountered in the wild. In addition to *TEPI,* the authors also found evidence of less intense positive selection acting on *TEPII.* In contrast, there was no evidence that *TEPIII* or *TEPIV* are evolving under positive selection.

In *Drosophila* larvae, *TEPI, TEPII, TEPIII, TEPIV* and *Mcr* are expressed in plasmatocytes and *TEPI, TEPII,* and *TEPIV* are expressed in the fat body, consistent with a role for theses genes in innate immunity. Several studies have shown that *TEPI-IV* and *Mcr* expression is upregulated after bacterial infection in larval hemocytes, larval fat body, and whole adult flies (Bou Aoun et al., 2011; Dionne et al., 2006; Irving et al., 2005; Lagueux et al., 2000). Additionally, transcriptome and QPCR analysis of gene expression in fly larvae following parasitoid wasp infection showed that *TEPI* is massively upregulated and may be important for the encapsulation and melanization of wasp eggs (Salazar-Jaramillo et al., 2014; Wertheim et al., 2005)

A large-scale RNAi screen in S2 cells found that Mcr is required for phagocytosis of the fungus *Candida albicans* (Stroschein-Stevenson et al., 2006). Interestingly, *Mcr* RNAi treatment of S2 cells did not affect phagocytosis of *E. coli* or *S. aureus.* Despite the lack of an active thioester motif, Mcr specifically binds to the surface of *C. albicans.* It does not, however, bind to another fungal pathogen, *S. cerevisiae*, indicating that Mcr

recognizes some feature unique to the *C. albicans* cell wall. The addition of conditioned media from untreated S2 cells to *Mcr* RNAi treated S2 cells is sufficient to rescue the *C. albicans* phagocytosis defect caused by the loss of *Mcr.* Hence, secreted Mcr is required to facilitate *C. albicans* phagocytosis in S2 cells. The same study also looked at role of TEPII and TEPIII in phagocytosis. *TEPII* RNAi led to a modest (about 25%) decrease in phagocytosis of *E. coli. TEPIII* RNAi had a similar effect on *S. aureus* phagocytosis.

More recently, a study was carried out to investigate the function of TEPs in the defense against pathogens. In brief, *TEPI - TEPIV* mutants were challenged with septic infections of Gram-positive bacteria (*S. pyogenes, S. aureus, E. faecalis,* and *L. monocytogenes*), Gram-negative bacteria (*E. coli* and *E. cloacae*), *Mycobacterium marinum,* or a fungal pathogen (*B. bassiana*) (Bou Aoun et al., 2011). Surprisingly, the *TEPI, TEPII, TEPIII, TEPIV* deficient flies were not more susceptible to the bacterial or fungal infections than wild type flies. A similar phenotype was observed in *TEPII/TEPIII* null double mutants and *TEPII/TEPIII/TEPIV* mutants (obtained by crossing the *TEPII/TEPIII* null double to a P-element insertion mutant). Finally, a modified *in vivo* adult phagocytosis conducted in triple *TEPII-IV* mutants showed that adult hemocytes of these flies were capable of phagocytosis *E. coli* pHrodo-labeled bioparticles. Based on these results, the importance of TEPs in adult flies immunity is uncertain (Bou Aoun et al., 2011). At this point, the relative contribution of TEPs and their role during opsonization of bacteria is still in question. The immune function of TEPs may be difficult to decipher *in vivo* if the effects of loss-of-function mutations are masked by other opsonins in *Drosophila.* To fully ascertain the role TEPs play in the cellular immune response, it would be instructive to assess phagocytosis of multiple microbes in

TEP mutants or in animals expressing *TEP* RNAi in hemocytes, fat body, or other tissues.

The best evidence that TEPs act as opsonins comes from studies in the mosquito, *Anopheles gambiae.* A family of 19 TEP genes has been identified in the genome of *A. gambiae* (Christophides et al., 2002). The most extensively studied TEP in mosquitos is *A. gambiae TEP1* (*aTEPI*). Similar to what was observed in *Drosophila,* expression of *aTEPI* increased after septic infection with a mixture of *E. coli* and *M. luteus* (Levashina et al., 2001). Importantly, western blotting analysis showed that aTEPI is detected at high levels in mosquito hemolymph and in conditioned media from a mosquito hemocyte cell line, 5.1. *In vivo,* the secreted aTEPI originates from mosquito hemocytes as immunofluorescence studies with an anti-aTEPI antibody found that the protein is selectively expressed in hemocytes throughout the body cavity. To assess the binding of aTEPI to bacteria, *E. coli* or *S. aureus* were incubated with 5.1 conditioned media, precipitated, and probed with the anti-aTEP1 antibody. aTEPI bound to both bacteria, and this binding was dramatically reduced when the aTEPI was chemically inactivated indicating that the aTEPI binds to bacteria in a thioester-dependent manner. Phagocytosis of *E. coli* by mosquito 5.1 cells was dramatically enhanced after the addition of conditioned media. This effect was lost when the added media was either pre-treated to chemically inactive thioester-containing proteins or when it was obtained from 5.1 cells treated with dsRNA that inhibited the expression of *aTEPI* in the cells. Similar effects were also observed in experiments carried out using two additional Gram-negative bacteria (*Serratia marcescens* and *Salmonella typhimurium*), but not in experiments testing the phagocytosis of Gram-positive bacteria (*Bacillus subtilis, M. luteus,* and *S.*

aureus) Thus, secreted aTEPI opsonizes and enhances the phagocytosis of Gram-negative bacteria by mosquito 5.1 cells. However, the authors noted that 5.1 cells showed only low levels of phagocytosis of Gram-positive bacteria. It is possible that 5.1 cells may simply be less capable of phagocytosis of Gram-positive bacteria.

The role of aTEPI in mosquitos was confirmed in a small-scale *in vivo* dsRNA screen (Moita et al., 2005). Down-regulation of aTEPI in adult mosquitos led to a 60% reduction in *E. coli* phagocytosis and a nearly 40% decrease in phagocytosis of *S. aureus.* Furthermore, *aTEPIII* down-regulation decreased *E. coli* phagocytosis by about 50%. *aTEPIV* dsRNA treatment had the most dramatic effect on phagocytosis, with a 60% reduction in phagocytosis of both pathogens. While *in vitro* studies from this group failed to detect the effect of silencing *aTEPI* on *S. aureus* phagocytosis, the studies described in Moita *et al.* found that silencing *aTEPI in vivo* decreased the uptake of the bacteria by adult hemocytes. These findings underscore the importance of *in vivo* experiments testing the function of immune genes.

An opsonin-like role has also been described for Dscam1, and soluble Dscam1 protein is present in S2 cell-conditioned medium and larval hemolymph. Thus, in addition to TEPs, Dscam1 may also function to opsonize microbes present in the hemolymph of the insect. However, the identity of receptors for specific isoforms of Dscam1 or TEPs on the surface of phagocytes has yet to be determined and could be an area for future study.

Additional work has found a role for several opsonin-like PRRs in mosquitos. The mosquito homolog of *Drosophila* Nimrod B2, *AgNimB2,* was shown to mediate phagocytosis of *S. aureus in vivo* (Midega et al., 2013). AgNimB2 has 7 NIM repeats

and is predicted to be secreted. Interestingly, biochemical analysis revealed that AgNimB2 does not directly bind to the surface of *S. aureus*. The authors suggest that AgNimB2 binds to opsonins like aTEP1 on the surface of bacteria and mediates the phagocytosis of opsonized bacteria by then binding to membrane-bound receptors on phagocytes.

C. Phagocytic Engulfment of Particles

Much of what is known about invertebrate innate immune signaling cascades has been acquired from studies of the humoral immune response. In comparison, relatively less is known about the signaling pathways that mediate phagocytosis in insect blood cells. Based on characterizations and observations of phagocytosis in mammalian systems, it is believed that ingestion of receptor-bound particles proceeds either through zipper-like movements of the plasma membrane around the particle, sinking of the particle into the cytoplasm, or through macropinocytosis (Figure 1-2) (Reviewed in (Swanson, 2008).

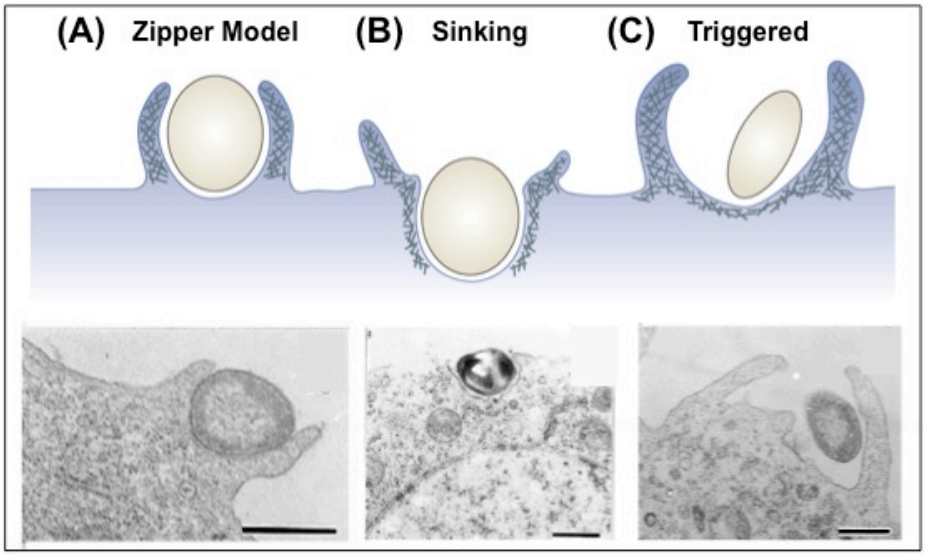

Figure 1-2: Models of plasma membrane movements and particle internalization during phagocytosis.

A. Zipper Model of phagocytosis; plasma membrane extensions form tight, receptor-mediated interactions with the particle. **B.** Sinking model of phagocytosis; the phagosome forms when the particle appears to sink into the cytoplasm. **C.** Triggered phagocytosis; bacteria are internalized by when they bind to receptors on the cell surface, triggering macropinosomes closure. Below each model is a transmission electron micrograph of *S. aureus* phagocytosis by a *Drosophila* larval hemocyte that matches the model of particle internalization depicted above. *Figure adapted from* (Swanson, 2008) and (Pearson et al., 2003)

In mammals, Fcγ-receptor mediated phagocytosis occurs in a zippering fashion (Griffin et al., 1975; Griffin et al., 1976). After binding to antibody-opsonized particles, Fcγ receptors cluster together and this triggers the activation of Src-family tyrosine kinases, which in turn phosphorylate and activate the antibody-bound receptor. Phosphorylation of tyrosine residues in the receptor leads to the recruitment of additional cytoplasmic proteins (lipid kinases, phosphatases, and adaptor molecules) to form a signaling complex that ultimately results in the stimulation of actin polymerization near the plasma membrane to create protrusions that extend over the particle. These protrusions, known as lamellipodium, are sheet-like extensions of the plasma membrane that contain branched actin networks. Lamellipodium spread over the opsonized particle in a receptor-guided, zipper-like fashion to form a tight phagocytic cup that, once closed, engulfs the particle into a nascent organelle called a phagosome.

Phagocytosis by receptors for complement-opsonized particles is morphologically distinct from Fcγ-receptor mediated phagocytosis. The complement receptor, CR3, is an α_M/β_2 integrin heterodimer that binds to iC3b-opsonized particles. Scanning electron microscopy of mammalian macrophages revealed that complement-opsonized particles appear to sink into phagosomes during C3R-mediated phagocytosis (Kaplan, 1977).

In contrast, engulfment of bacteria via macropinocytosis is not directly guided by close interactions of the membrane and target particle. Instead, macropinosomes form spontaneously at the cell surface or in response to stimulation of growth-factor receptors. Membrane ruffles, sheet-like extensions of the plasma membrane that form via actin filament assembly, sometimes curve into cavernous, open cups at the cell surface. Ruffle closure creates a large fluid-filled vesicle that is delivered to the endosomal pathway in

the cytoplasm. Pathogens, such as *Salmonella typhimurium, Legionella pneumophila,* and Vaccinia virus, and apoptotic cells bind to receptors found on the cell surface within the open macropinosome cup; stimulating cup closure and particle uptake (Alpuche-Aranda et al., 1994; Hoffmann et al., 2001; Mercer and Helenius, 2008; Watarai et al., 2001). Phagocytosis via macropinosomes is also called triggered phagocytosis.

A seminal study by the Ezekowitz group performed an ultrastructural examination of phagocytosis in S2 cells, mbn-2 cells, and larval plasmatocytes (Figure 2) (Pearson et al., 2003). Phagocytosis of FITC-labeled *S. aureus* primarily occurs through the zippering of the plasma membrane around the bacteria. Interestingly, the study also found evidence that *S. aureus* phagocytosis also occurs via macropinocytic-type engulfment as well as by sinking into the cell, like macrophage complement-mediated phagocytosis. The observation of zippering-type phagocytosis indicates that receptor clustering and activation may be of primary importance for *S. aureus* phagocytosis in the fruit fly. Additionally, the observation of macropinocyte-like phagocytosis, indicates that receptors within macropinosomes are capable of triggering phagocytosis without the formation of a tight phagocytic cup. Opsonized *S. aureus* particles may sink into the cell in a manner akin to mammalian CR3-mediated phagocytosis. The study provided valuable insight into the dynamics of plasma membrane changes as well as the underlying cytoskeletal alterations that accompany phagocytosis in *Drosophila.* Overall, the morphology of phagocytosis in *Drosophila* hemocytes is reminiscent of mammalian phagocytes and this study was key to establishing *Drosophila* hemocytes as a model to study phagocytosis *in vivo.*

Signaling from bound phagocytic receptors triggers coordinated rearrangements of the actin cytoskeleton. Small GTPases of the Ras superfamily, such as the Rho-GTPases Cdc42, Rac1, and Rac2 are recruited to the plasma membrane, where they associate with membrane phospholipids and proteins. Rho-GTPases function as molecular switches that alternate between active (GTP-bound) and inactive (GDP-bound) states. They are activated by guanine nucleotide exchange factors (GEFs), which facilitate the binding of GTP, and are inhibited by the hydrolysis of GTP, which is carried out by guanine nucleotide dissociation inhibitors (GDIs). The *Drosophila* gene *Ziziman-related* (*Zir*) is a Rho-GEF that interacts genetically with Cdc42 and Rac2 to mediate larval hemocyte phagocytosis of *E. coli* and *S. aureus* (Sampson et al., 2012). The primary function of Rho-GTPases during phagocytosis is the regulation and activation of cytoskeletal remodeling enzymes. Rac1 activates WAVE, a member of the Wiskott-Aldrich syndrome protein (WASP) family. WAVE then activates the Arp 2/3 complex, which stimulates actin nucleation, the initial step required for the formation of new actin filament structures. Cdc42 activates WAS(p), the founding member of the WASP family, which in turn activates the Arp 2/3 complex. Cofilin and cofilin-like proteins control the debranching and disassembly of actin filaments to facilitate recycling of actin monomers and structural changes necessary for cytoskeletal reorganization (Chan et al., 2009). Cdc42, Rac1, Rac2 and the Arp 2/3 complex were all identified in RNAi screens and genetic studies to find factors that mediate phagocytosis in S2 cells (Agaisse et al., 2005; Philips et al., 2005; Stroschein-Stevenson et al., 2006; Stuart et al., 2005).

To understand how Rho-GTPases control phagocytosis of bacterial pathogens in *Drosophila* hemocytes, the Faurvaque group generated transgenic *Drosophila* mutants

that expressed the Gram-negative pathogen *Pseudomonas aeruginosa* exotoxin, ExoS, specifically in hemocytes (Avet-Rochex et al., 2005). ExoS contains an N-terminal GTPase activating (GAP) domain that inactivates Rho-GTPases and Rho-dependent signaling. Expressing ExoSGAP in blood cells led to significantly reduced *E. coli* uptake by both adult and larval hemocytes. It is interesting to note, that the ExoS may inhibit the fly cellular response, preventing phagocytosis of *P. aeruginosa* by hemocytes. The same group also explored potential immune contribution of individual Rho-GTPases, Rho1, Rac1, Rac2, and Cdc42 by testing mutant fly resistance to *P. aeruginosa* (Avet-Rochex et al., 2007). The only mutants to show significant susceptibility to *P. aeruginosa* were the *Rac2*-deficient flies. Furthermore *Rac-2* mutants were also more susceptible to infection with other Gram-negative (such as *E.* coli) and Gram-positive (*E. faecilis* and *S. aureus*) bacterial pathogens. Larval hemocytes from *Rac2* mutants showed a 35% decrease in uptake of *E. coli* and a 55% decrease in *S. aureus* phagocytosis.

Studies of Draper-mediated phagocytosis have provided the most complete picture of intracellular signaling cascades that take place during *Drosophila* phagocytosis. During *S. aureus* phagocytosis, Draper signals through Rho-GTPases, Rac1 or Rac2 (Hashimoto et al., 2009). Larval hemocytes from flies with loss of one copy of *draper, Rac1,* or *Rac 2* show no phagocytosis defects. However, hemocytes from flies with simultaneous heterozygous loss of *draper, Rac1,* or *Rac 2* were dramatically impaired for bacterial phagocytosis. Thus, after Draper binds *S. aureus* ligands, Rac 1 and/or Rac2 are required for the engulfment of the microbe. The cytoplasmic signaling complex that controls Draper-mediated phagocytosis of apoptotic cells has been examined using classical genetic approaches (Ziegenfuss et al., 2008). In *Drosophila* glial

cells, Draper physically interacts with Shark, an SH2 domain containing non-receptor tyrosine kinase that is similar to mammalian Zap-70, and this interaction is dependent upon the Src-family kinase member, Src42A. Based on genetic and biochemical studies, Ziegenfuss and colleagues proposed the following model of signaling during Draper-mediated apoptotic cell clearance: Draper binds to target ligands on cell corpses, Src42A phosphorylates tyrosines located in the intracellular ITAM motif of Draper, SH2 domain of Shark associates with the Draper's phosphorylated ITAM domain, Shark activates further downstream signaling events required for apoptotic cell uptake. To determine if Shark plays a role in Draper-mediated uptake of *S. aureus,* Hashimoto and colleagues generated a fly line with one copy of mutated alleles for both genes (Hashimoto et al., 2009). Larval hemocytes from single- and double-heterozygous flies showed no difference in the uptake of *S. aureus,* indicating that Shark does not act downstream of Draper to mediate uptake of *S. aureus*.

A number of genetic screens and RNAi screens have been conducted to identify proteins that regulate actin cytoskeleton reorganization during phagocytosis in the fruit fly. A forward genetic screen identified the *Drosophila* homolog of WAVE, D-SCAR, as an important regulator of *E. coli* and *S. aureus* phagocytosis in *Drosophila* larval hemoctyes (Pearson et al., 2003). The study also analyzed the role of the *Drosophila* WAS(p) (D-WAS(p)) homolog. *D-WAS(p)* RNAi specifically led to decreased *S. aureus* uptake by S2 cells. The differences observed after loss of D-SCAR or D-WAS(p) may indicate that these proteins function in independent pathways, perhaps downstream of receptors with distinct ligand specificity. An increase in *E. coli* and *S. aureus* phagocytosis was observed in larval hemocytes obtained from a line with a P-element

insertion in *chickadee,* the gene encoding the *Drosophila* homolog of profilin. Profilin sequesters free actin, and loss of profilin in *chic* mutants may lead to increased phagocytosis due to the higher availability of free actin and increased spontaneous actin nucleation. Loss of profilin also leads to decreased phagocytosis of *M. fortuitum*, demonstrating that profilin is a host factor required for general phagocytosis in *Drosophila* (Philips et al., 2005).

D. Phagosome Maturation

The process of particle internalization culminates in the formation of a membrane bound vesicle - the phagosome -which contains the microbe or cell corpse. Phagosome formation is followed by rapid series of biochemical and cellular changes that convert the nascent phagosome into a potent microbicidal and acidic organelle (Desjardins et al., 1994). Almost immediately, newly formed phagosomes undergo a series of highly ordered fusion and fission events with components of the endosomal pathway. This process, termed phagosome maturation, produces a highly acidic and hydrolytic phagolysosome designed to destroy the cargo (Kinchen and Ravichandran, 2008). The maturation of phagosomes involves interactions with other cellular organelles, including early endosomes, recycling endosomes, late endosomes and lysosomes (Vieira et al., 2002). The stages of phagosomal maturation in mammalian cells are illustrated in Figure 1-3. A general overview of the topic is discussed below, with specific details regarding *Drosophila* homologs of the following components: Rab GTPases, phosphatidylinositol 3-kinase, Vacuolar H+-ATPase, the Endosomal sorting complex required for transport (ESCRT) complex, the Vacuolar protein sorting-C complex.

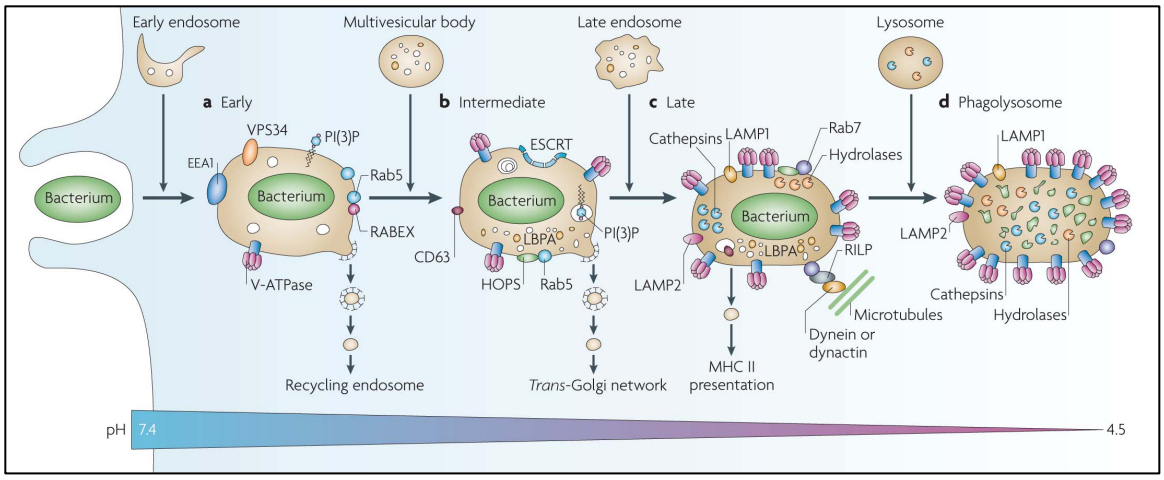

Figure 1-3: The stages of phagosome maturation.

The phagosome travels along microtubules towards the center of the cell. Along the way, it interacts with compartments of the endocytic pathway, undergoing a progressive acidification that culminates in the formation of a phagolysosome. *Figure taken from* (Flannagan et al., 2009).

Phagosomes formed by receptor-mediated particle internalization quickly fuse with early endosomal vesicles (Mayorga et al., 1991). The small Rab GTPase, Rab5, coordinates early endosomal targeting, tethering and fusion with the nascent phagosome (Bucci et al., 1992). Rab5 is recruited to newly formed phagosomes by the GTPase Dynamin (Kinchen et al., 2008). Overexpression of Rab5 in *Drosophila* hemoctyes leads to an accumulation of *E. coli*-containing vesicles in larval hemocytes (Horn et al., 2014). Thus, altered levels of Rab5 inhibit the process of phagosome maturation in *Drosophila* hemocytes. Rab5 recruits multiple effectors to the early endosomal/phagosomal membrane, including the early endosome antigen 1 (EEA1), SNARE proteins (which are

required for membrane fusion), and Vps34 and its regulatory subunit, Vps15 (also known as p150).

Vps15 is a serine-threonine kinase that recruits Vps34 to the early phagosome. Vps34 is a class III phosphatidylinositol-3 kinase (PI3-kinase) that generates phosphatidylinositol-3-phosphate (PI(3)P) on the early phagosomal membrane (Vieira et al., 2001). PI(3)P interacts with proteins containing FYVE (for conserved in **F**ab1, **Y**OTB, **V**ac1, and **E**EA1) domains. The *Drosophila* homolog of mammalian Vps34, Phosphotidylinositol 3 kinase 59F (Pi3K59F) functions during the cellular immune response to bacterial and fungal pathogens (Qin et al., 2011; Qin et al., 2008). Similar to its counterpart in mammals, *Drosophila* homolog of EEA1, Rabenosyn-5, is a FYVE domain-containing protein that binds to PI(3)P and Rab5 on the surface of the phagosome, where it is required for fusion of endocytic vesicles and early endosomes (Morrison et al., 2008; Simonsen et al., 1998). The generation of PI(3)P is essential for the progression of phagosome maturation. In mouse fibroblasts, PI(3)P stabilizes the interaction of EEA1 on the early phagosome. Loss of PI3-kinase activity leads to decreased association of EEA1 and blockage of phagosome maturation (Vieira et al., 2001)

In eukaryotes, Vps15 is known for its role in endocytosis and phagocytosis as part of the PI3-kinase complex with Vps34. In an effort to identify genes that regulate activation of the *Imd* pathway after *E. coli* infection, the Wu group conducted a forward genetic screen of EMS mutant flies. This screen identified a *Drosophila* Vps15 mutant, *ird,* as important for IMD pathway activation (Wu et al., 2007). *ird1* mutants were shown to be more susceptible to infection with *E. coli* or *M. luteus,* and had impaired

antimicrobial peptide synthesis in the mutant which may account for this effect. It is also possible that *ird1* mutants are defective for phagosome maturation of bacteria, but this has not been experimentally verified. The *Drosophila* Vps34/Vps15 complex was recently implicated in vesicle trafficking during stress-induced autophagy, a process that, like phagosome maturation, requires coordinated vesicle-trafficking pathways (Anding and Baehrecke, 2015). Analysis of phagosome maturation phenotypes of *ird1* mutants may provide evidence of a role for Vps15 in the cellular immune response in the fruit fly.

The vacuolar H+-ATPase (V-ATPase) complex is found on the phagosome membrane at very early stages and is required to acidify the phagosomal lumen during phagosome maturation (Beyenbach and Wieczorek, 2006). In *Drosophila,* the V-ATPase complex is made up of multiple subunits. Several V-ATPase subunits have been implicated in the immune response in the fruit fly. The Perrimon group identified three components of the V-ATPase in a genome-wide RNAi screen looking for genes that altered the expression of GFP from the *map24* promoter of *M. fortuitum* (Philips et al., 2005). The *map24* promoter is responsive at low pH, silencing of V-ATPase components increases the pH of the lumen of the phagosome, thereby decreasing GFP expression under the *map24* promoter. Additionally, eight V-ATPase subunits were identified in a genome-wide S2 cell RNAi screen for genes that are important for the pathogenesis of the facultative intracellular Gram-positive bacteria, *Listeria monocytogenes* (Cheng et al., 2005). RNAi of individual V-ATPase subunits led to fewer infected cells at 7.5 hours post infection. *Listeria* exits the phagosome to replicate in the host cell cytosol using the pore forming toxin listeriolysin O (LLO) and phospholipase C. LLO functions at low pH (~5.5) and loss of the V-ATPase subunits effectively halted the acidification of the

phagosome, impairing the function of LLO and the vacuolar escape mechanism of the bacteria. It has also been proposed that the V-ATPase complex promotes fusion between Zebrafish phagosomes and lysosomes but a similar role has yet to be uncovered in *Drosophila* hemocytes (Peri and Nusslein-Volhard, 2008).

During the transition from the early to the late phagosome stage, multivesicular bodies (MVB) begin to appear within the phagosome. MVBs are luminal vesicles that arise from inward budding and scission of portions of the limiting membrane of endosomes and phagosomes. In the endosomal pathway, transmembrane proteins that are destined for degradation are ubiquitinated and then sorted into MVBs (Lee et al., 2005). The ubiquitin tags are recognized by the ESCRT complex, which then sorts the tagged proteins into MVBs. Late phagosomes and endosomes also contain lysobisphosphatidic acid (LBPA)(Kobayashi et al., 1999). LBPA is a unique lipid found in MVBs .

Work from the Perrimon group illustrated a role for the ESCRT complex in the restricting the intracellular growth of *Mycobacterium* species in the fruit fly. Double-stranded RNAs targeting ESCRT factors *Vps28, CG8055, Tsg101* and *Vps4* led to a decreased induction of GFP expression under the pH responsive *map24* promoter in *M. fortuitum.* Unlike *peste* RNAi, which led to decreased *M. fortuitum* uptake, dsRNA targeting ESCRT impaired the formation of MVBs and effectively halted the phagosome maturation process at a stage that was not permissive for the induction of *map24* (Philips et al., 2008; Philips et al., 2005). The group then examined how silencing of ESCRT factors affects bacterial growth of the non-pathogenic *M. smegmatis* (Philips et al., 2008). S2 cells normally restrict the growth of *M. smegmatis,* but silencing of the ESCRT factors led to an increase in bacterial growth. These results indicate that the knockdown created a

permissive phagosome environment allowing *M. smegmatis* to survive, grow, and disseminate to other cells. ESCRT mediated sorting of ubiquitinated proteins is nearly absent when S2 cells are treated with dsRNA targeting ESCRT components *Tsg101, Vps28, CG8055,* or *Vps4,* as is evidenced by an accumulation of ubiquitin in the vesicular compartments in treated cells. In ESCRT depleted S2 cells, *M. smegmatis* colocalized with vesicles containing access ubiquitin, revealing that the ESCRT complex normally functions within phagosomes that contain bacteria. The ESCRT machinery works in an analogous manner in mammalian cells. RNAi depletion of *Tg101* and *Vps28* in the mammalian macrophage cell line, RAW267.7, led to significantly higher *M. smegmatis* growth and increased ubiquitin in bacteria containing phagosomes.

Four components of the ESCRT complex were identified in a genome-wide S2 cell RNAi screen looking for genes that are important for *Listeria* pathogenesis (Cheng et al., 2005). As previously stated, the pore forming toxin listeriolysin O (LLO) and phospholipase C are required for *Listeria* to escape the phagosome. To identify host genes that bypass the need for LLO in vacuolar escape, RNAi-treated S2 cells were infected with *Listeria* mutants lacking LLO (LLO-minus). LLO-minus mutant *Listeria* cannot normally escape from the phagosome. RNAi of ESCRT components *Tgs101, SNF7, Vps4, and Bro1* allowed LLO-minus bacteria to escape from the phagosome. However *Listeria* mutants lacking both LLO and PLC were not able to escape the phagosome of ESCRT-depleted cells. In wild type *Listeria,* LLO-mediated escape occurs during the MVBs/late endosome stage, when the pH of the phagosomal lumen is slightly acidic (~5.5). Absence of ESCRT components prevents the formation of MVBs, and may allow LLO-minus mutants to escape from early phagosome using PLC alone.

After the formation of MVBs the phagosome transitions to the late stage, which is characterized by a more acidic lumenal pH. The late phagosome is characterized by the presence of several molecules including lysosomal-associated membrane proteins (LAMPs) and hydrolases. In mammalian cells, LAMP proteins are required for the last stage of phagosome maturation, the fusion of the phagosome with the lysosome (Huynh et al., 2007). A recent study in *Drosophila* to identify host factors that are required for phagocytosis and intracellular maintenance of the protozoan parasite *Leishmania donovani* found that *L. donavani* amastigotes colocalize with vesicles that are positive for *Drosophila* Lamp1 (DmLamp1 formerly known as CG3305) within S2 cells (Peltan et al., 2012). As proof of concept that *L. donovani* can infect S2 cells, the authors carried out immunofluorescence studies of infected S2 cells expressing a DmLamp1-GFP fusion protein. Late-stage *L. donovani*-containing phagosomes are positive for DmLamp1 indicating that vesicles containing the protozoan are trafficked undergo phagosome maturation within S2 cells. The relative importance of DmLamp1during the cellular immune response to bacterial pathogens should be assessed in *DmLamp1* loss-of-function mutant flies to more fully characterize for the importance of Lamp1 during the process of phagocytosis and phagosome maturation *in vivo*.

In addition to the acquisition of LAMPs, additional V-ATPases are acquired by the late phagosomes. Furthermore, the vesicles acquire the small Rab-GTPase Rab7, which is a characteristic marker of late phagosomes (Desjardins et al., 1994). Rab7 is key to membrane trafficking between phagosomes and late endosomes or lysosomes and it recruits effectors such as Rab-interacting lysosomal protein (RILP), which tethers the vesicle to the dynein-dynactin motor, facilitating the movement of the phagosome

towards the center of the cell (Harrison et al., 2003; Jordens et al., 2001). Vacuolar protein sorting-C (VPS-C) complexes are vital players that interact with SNAREs and Rabs during the phagosome maturation process. There are two distinct vacuolar protein sorting C complexes: CORVET (class C core vacuole/endosome tethering) and HOPS (homotypic fusion and vacuole protein sorting) (Reviewed in (Balderhaar and Ungermann, 2013; Solinger and Spang, 2013). The CORVET complex interacts with Rab5-GTP and promotes early endosome/phagosome fusion. The HOPS complex interacts with Rab7-GTP on late endosomes/MVBs to facilitate their fusion with lysosomes. Additionally, live cell imaging studies in a human cell line indicates that the HOPS complex exchanges Rab5 for Rab7 to facilitate the transition from early to late phagosomes (Rink et al., 2005). Much of the early work defining the composition of each complex was carried out in yeast. Both complexes are heterohexameric: they are composed of four shared class C subunits (Vps11, Vps16, Vps18, and Vps33) and two Rab-specific subunits. In *Drosophila,* both Vps33 and Vps16 have two homologs (*car* and *Vps33B, Vps16A* and *Vps16B,* respectively) (Li and Blissard, 2015; Pulipparacharuvil et al., 2005). Vps16A and Vps16B are predicted to associate with different HOPS complexes, and this association may dictate the function of the complex (Pulipparacharuvil et al., 2005). *Vps16A*-mutant *Drosophila* larvae are unable to clear autophagosomes following starvation-induced autophagy, indicating that the Vps16A subunit of HOPS is essential for the fusion of autophagosomes with lysosomes (Takats et al., 2015). The other *Drosophila* Vps16 homolog, Vps16B, has been implicated in phagosome maturation (Akbar et al., 2011). Vps16 mutants, *full of bacteria* (*fob*), are highly susceptible to non-pathogenic *E. coli* and, survival of *fob*-null flies can be rescued

by specifically expressing wild type *fob* in hemocytes. *fob* adult and larval hemocytes are able to engulf *E. coli* but show defects in phagosome acidification. *fob* mutant hemocytes show no defects in the acquisition of early endosome markers such as Rab5 and Rbsn-5, but have significantly higher numbers of Rab-7-positive phagosomes, suggesting that phagosome maturation is stalled at this stage. To test if *fob* mutant hemocytes have defective fusion phagosome/lysosome fusion, wild type and mutant hemocytes were treated with Alexa-488 labeled dextrans, which, when internalized by fluid-phase endocytosis, labels lysosomes. The hemocytes were then challenged with fluorescein-labeled *E. coli* and co-localization of dextran and bacteria was examined using immunofluorescence. Approximately 30% of bacteria-positive phagosomes co-localized with dextran-labeled lysosomes in wild type hemocytes. Fewer, only 9%, of bacteria-containing phagosomes co-localize with lysosomes in *fob* mutant hemocytes. These results confirm that Vps16B mediates phagosome to lysosome fusion in *Drosophila.*

The final step in the maturation process is the formation of the phagolysosome (pH~4.5). Phagolysosomes are highly effective microbicidal organelles that are equipped with host factors that impede microbial growth while simultaneously attacking and degrading the pathogen. Key cofactors of bacterial housekeeping enzymes (such as free iron or divalent metal ions (Fe^{2+}, Zn^{2+}, and Mn^{2+})) are removed from the phagosomal lumen to prevent bacterial growth. Free iron is sequestered by lactoferrin, a glycoprotein found in the phagosome lumen while divalent metal ions are actively removed from the phagosome by NRAMP, an integral membrane protein that extrudes the ions from the phagosomal lumen. Reactive oxygen and nitrogen species attack bacterial DNA, proteins, and lipids to destroy the pathogen. Reactive oxygen species (ROS) are generated through

the action of the membrane bound NOX2 NADPH oxidase, which transfers electrons from cytosolic NADPH to molecular oxygen (O_2^-) and releases the O_2^- into the phagosomal lumen. Superoxide dismutase (SOD) catalyzes the dismutation of O_2^- into H_2O_2, which in turn can be converted into additional toxic ROS species (hypochlorous acid and chloramines) that kill microoganisms. Reactive nitrogen species (RNS) are also important antimicrobial factors. The enzyme inducible nitric oxide synthase, iNOS, catalyzes the formation of nitric oxide on the cytoplasmic side of the phagosome. Nitric oxide diffuses across the bilayer into the phagosome, where it encounters ROS and converts to various RNS that are highly toxic to the microorganism. Phagolysosomes are also equipped with an assortment of bactericidal elements: antimicrobial peptides (such as membrane permeablizing defensins), peptidases (such as cysteine protease, aspartate proteases, serine proteases), lipases and hydrolases. Overall, the phagosome is a very dynamic structure that changes by acquiring different proteins during maturation, ultimately becoming a highly microbicidal and degradative organelle. Studies in cell lines and model organisms have identified proteins that participate in the maturation process as well as antimicrobial effectors that destroy the cargo within the organelle. Proteomic studies to elucidate the protein composition of the phagosome have provided insight into the structure and function of the organelle. In particular, proteomic analyses of latex bead containing phagosomes in humans and S2 cells, have highlighted the complexity of the phagosome, generating large-scale interaction networks that have provided the basis for functional studies (Garin et al., 2001; Stuart et al., 2007).

The Phagosome Proteome

Using SDS gel electrophoresis followed by tandem mass spectrometry, the Ezekowitz group identified 617 proteins associated with latex-bead-containing S2 cell phagosomes (Stuart et al., 2007). Among the proteins identified in phagosome were components of the exocyst, a octameric protein complex involved in tethering vesicles to the plasma membrane prior to exocytosis. The exocyst is made up of eight components (Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70, and Exo84), six of which were identified in the S2 cell phagosome. Prior to this study, the exocyst was not known to play a role in phagocytosis. The authors examined how RNAi-mediated silencing of exocyst components affected phagocytosis of *E. coli* and *S. aureus*. Silencing of Sec8, Sec10, and Sec18 decreased uptake of both microbes. Exo70 RNAi impaired the uptake of *E. coli* while Sec3 affected uptake of *S. aureus*. As part of the secretory pathway, Sec3 and Exo70 localize to target membranes and provide docking sites for other exocysts components. The authors hypothesized that depending on the microbes encountered and the receptors engaged, receptor-specific Rho-GTPases recruit either Exo70 or Sec3. Exo70 or Sec3 then provide docking site for the Sec8, Sec10, and Sec15 exocyst components, thus facilitating the delivery of endosomes to the phagocytic cup.

The Ezekowitz group also identified several Rab-GTPases within the S2 cell phagosome proteome. To understand how these Rab-GTPases function during the process of phagosome maturation, the Wu group conducted a phagosome maturation screen of adult flies expressing dominant negative forms of each of the Rabs identified in S2 cell phagosomes (Garg and Wu, 2014). They found that hemocytes expressing a dominant negative form of Rab14 showed significantly impaired *S. aureus* phagosome

maturation. Rab14 is found on early endosome containing Rab5 and late endosomes containing Rab7 and could work in conjunction with both Rab5 and Rab7 to modulate phagosome maturation. Importantly, Rab14-null mutant flies do not show defects in uptake of *E. coli* or *S. aureus* heat-killed bioparticles labeled with fluorescein. However, the maturation of *S. aureus* or *E. coli*-containing phagosomes was impaired in Rab14-null mutants and this effect could be rescued by expressing wild type Rab14 isoforms specifically in hemocytes.

The authors also found that *Rab14* mutant cells showed significantly less recruitment of Rab7 and the lysosomal marker Spinster onto *S. aureus*-containing phagosomes. Interestingly, they also found that *Rab14* mutants were not susceptible to *E. coli* infection but were extremely susceptible to *S. aureus* infection and this defect was associated with a higher bacterial load in *S. aureus* infected *Rab14* mutant flies. Thus, Rab14 is important during the *S. aureus* phagosome maturation process in *Drosophila.* In the absence of Rab14, *S. aureus* phagosome maturation is not completely abolished, but instead shows delayed kinetics. This defect could be attributed to impaired or delayed recruitment of Rab7 to phagosomes in Rab14 mutant cells. This work demonstrates the importance of the functional characterization of phagosome components in relation to the immune response *in vivo.*

III. The Humoral Immune Response

A. Humoral immune signaling cascades

Drosophila combats pathogens through both cellular and humoral immune responses. The humoral, or systemic, immune responses is well characterized in *Drosophila.* The hallmark of humoral immunity is the synthesis and secretion of antimicrobial peptides (AMPs), small peptides that exhibit a broad spectrum of antimicrobial activity against bacteria and other pathogens (Bulet et al., 1999; Izadpanah and Gallo, 2005).Two Nuclear Factor-*κ*B (NF-*κ*B) signaling pathways, the Toll and IMD pathways, regulate the induction of AMPs. NF-*κ*B/Rel proteins are a class of transcription factors that are related through the highly conserved N-terminal Rel homology domain (RHD). The RHD contains sequences that mediate DNA binding, dimerization, and nuclear localization. The *Drosophila* genome contains three genes that encode NF-*κ*B factors: *Dorsal, Dif* (*Dorsal-related immunity factor*)*,* and *Relish.* These NF-*κ*B factors are the master regulators of the humoral immune response. Gram-positive bacteria, fungal pathogens, and Drosophila X virus activate the Toll pathway, which in turn activates *Dif* and *Dorsal,* resulting in the induction of target genes such as the AMP *Drosomycin* (Lemaitre et al., 1996; Michel et al., 2001a; Zambon et al., 2005) The IMD signaling pathway responds to DAP-type PGN from Gram-negative bacteria, leading to the activation of Relish, which induces the production of immune responsive genes such as the AMPs *Diptericin, Attacin* and *Cecropin* (Hedengren et al., 1999; Lemaitre et al., 1995; Leulier et al., 2000).

The Toll Pathway

The Toll pathway was first described for its role regulating dorsal-ventral polarity during *Drosophila* embryonic development (Belvin and Anderson, 1996; Nusslein-Volhard and Wieschaus, 1980). Subsequent studies found that the Toll pathway is a conserved component of the innate immune response in both insects and humans (Lemaitre et al., 1996; Medzhitov et al., 1997). The structure and function of mammalian TLRs has been extensively studie and is reviewed in (Izadpanah and Gallo, 2005) The *Drosophila* Toll signaling pathway is similar to the mammalian TLR/IL-1R signaling pathway.

The Toll pathway is initiated when Lys-type PGNs from the cell walls of Gram-positive bacteria are sensed by PGRP-SA and PGRP-SD (Bischoff et al., 2004; Gottar et al., 2002; Michel et al., 2001b). Lys-type PGN is also recognized by a third secreted protein, Gram-negative binding protein (GNBP1) (Gobert et al., 2003; Pili-Floury et al., 2004). Fungi also induce Toll signaling when the receptor GNBP3 recognizes *β*-(1, 3)-glucan from the fungal cell wall (Gottar et al., 2006). Thus Lys-type PGN recognition by a complex of PGRP-SA/SD/GNBP1 and *β*-glucan recognition by GNBP3 activate the Toll signaling pathway. Unlike its mammalian counterpart, the Toll receptor in *Drosophila* does not directly bind microbial ligands. Instead, once microbial ligands are recognized by PGRP-SA/SD/GNBP1 or GNBP3, a serine protease cascade is immediately activated that culminates in the activation of the Toll receptor ligand Spätzle. After PGN recognition by PGRP-SA/SD/GNBP1 the receptor complex is bound by the serine protease, Modular Serine Protease (ModSP) ((Buchon et al., 2009; Kim et al., 2008). ModSP initiates a protease cascade that activates the serine proteases Grass

and Spätzle processing enzyme (SPE) (El Chamy et al., 2008; Kambris et al., 2006). SPE directly cleaves the protein pro-Spätzle into activated Spätzle, the direct ligand for the Toll receptor (Jang et al., 2006; Schneider et al., 1994; Shia et al., 2009; Weber et al., 2003). Additionally, virulence factors released by some fungi, such as the PR1 protease of entomopathogenic fungi, trigger the Toll pathway by activating the serine protease Persephone (El Chamy et al., 2008; Gottar et al., 2006; Ligoxygakis et al., 2002; St Leger et al., 1992). Persephone activates the same SPE used by the PGRP/GNBP pathways.

Once Spätzle binds to the Toll receptor, the receptor homodimerizes and activates intracellular signaling (Hu et al., 2004; Mizuguchi et al., 1998; Weber et al., 2003). Activated Toll signals through its TIR domain to recruit the adaptor protein, MyD88, which then forms a heterotrimeric complex with the adaptor protein Tube and the kinase Pelle (Horng and Medzhitov, 2001; Sun et al., 2002; Tauszig-Delamasure et al., 2002). Once recruited to the complex, Pelle is activated and phosphorylates Cactus, an IκB-like protein that sequesters Dif and Dorsal in the cytoplasm (Sun et al., 2004). Cactus is subsequently degraded and Dif and Dorsal translocate into the nucleus, where they activate the transcription of AMP genes (Wu and Anderson, 1998).

The IMD Pathway

The IMD pathway is homologous to the mammalian Tumor necrosis factor-α (TNF-α) receptor signaling pathway. The IMD signaling pathway is triggered by the detection of DAP-type PGN by PGRP-LC or PGRP-LE (Gottar et al., 2002; Takehana et al., 2002). PGRP-LC is a membrane bound receptor and PGRP-LE is a cytosolic PGRP receptor that recognizes microbial ligands found within the cytoplasm (Kaneko et al., 2006; Yano et al., 2008). Once PGRP-LC or PGRP-LE detect DAP-type PGN, they

recruit the adaptor protein, IMD to their N-terminal RHIM-like (receptor-interacting protein homotypic interaction motif) domain (Choe et al., 2005; Georgel et al., 2001; Kaneko et al., 2006). IMD has a central role in the pathway as it functions as a scaffold for the initiation of the signaling cascade that culminates in the phosphorylation and activation of the NF-*κ*B transcription factor Relish.

IMD recruits the adaptor protein, FADD (Fas-associated death domain), via a homotypic death domain interaction (Naitza et al., 2002). FADD then interacts with the caspase DREDD (Death-related ced-3/Nedd2-like protein) and this interaction is critical for downstream signaling events (Hu and Yang, 2000; Leulier et al., 2000). IMD is cleaved in a DREDD-dependent manner, exposing a highly conserved IAP-binding motif (IBM) (Paquette et al., 2010). The IBM motif allows IMD to associate with *Drosophila* inhibitor of apoptosis 2 (DIAP2). DIAP2 contains a C-terminal RING domain that is essential for the robust ubiquitination of IMD that occurs after DIAP2 and IMD associate (Huh et al., 2007). Polyubiquinated IMD serves as a scaffold for TAK1 (transforming growth factor-β-activated kinase 1), which in turn activates the I*κ*B kinase (IKK) complex (Kleino et al., 2005; Silverman et al., 2003; Vidal et al., 2001). The IKK signaling complex is made up of the catalytic subunit of IRD5 (immune-response deficient 5; the *Drosophila* homolog of mammalian IKKβ) and a regulatory subunit Kenny (the fly homolog of mammalian IKKγ) (Lu et al., 2001). The IKK complex then phosphorylates several sites of the N-terminal portion of Relish (Silverman et al., 2000). Phosphorylated Relish is then cleaved to release the inhibitory C-terminal domain (Rel-49), thus allowing the N-terminal Rel domain (Rel-68) to translocate into the nucleus to activate the transcription of AMP genes, such as *Diptericin* and *Cecropin*. Cleavage of

IMD is a caspase-mediated process, and it has been proposed that DREDD might directly cleave phosphorylated IMD (Stoven et al., 2000; Stoven et al., 2003).

IV. Interactions between the Cellular and Humoral Responses

Several studies have demonstrated that humoral and cellular immune responses work together to combat bacterial infections in the fruit fly (Braun et al., 1998b; Brennan et al., 2007; Matova and Anderson, 2006; Nehme et al., 2011; Pham et al., 2007). In one study, *imd* mutant flies succumbed to an otherwise harmless Gram-negative *Escherichia coli* infection only when the cellular immune response is ablated by pre-injection with latex beads (Elrod-Erickson et al., 2000). Loss of *Psidin,* a lysosomal protein, impairs phagosome maturation of Gram-positive and Gram-negative bacteria in hemocytes, but also leads to deficient induction of the AMP gene *Defensin* in the fat body (Brennan et al., 2007). This type of crosstalk is critical for an effective host response against a multitude of bacterial pathogens. However, experiments in fruit flies as well as in another insect model, the beetle *Tenebrio molitor*, indicate that hemocyte-mediated defenses, and not the humoral immune response, are the most immediate and critical component of the immune response to *S. aureus* (Haine et al., 2008; Nehme et al., 2011). In *Tenebrio molitor*, hemocytes effectively clear 99.5% of *S. aureus* within an hour of infection. The humoral immune response is induced after the majority of the bacteria are eliminated by hemocytes, and AMPs control the growth and dissemination of remaining pathogens (Haine et al., 2008). Thus, in both insects and humans, blood cell recognition and lysosomal degradation of internalized microbes is essential to combat *Staphylococcus aureus* infections.

V. New resources to study phagocytosis in *Drosophila*

A. The *Drosophila* Genetic Reference Panel

In 2012, a collection of 200 wild-derived inbred lines called the *Drosophila melanogaster* Genetic Reference Panel (DGRP) was made available by the Mackay laboratory (Mackay et al., 2012). These lines were derived from single mated females from a natural population in Raleigh, North Carolina. As proof of concept of the utility of using the DGRP to conduction phenotype-genotype studies, a subset of 40 lines were sequenced and assessed for a variety of quantitative traits such as chill coma recovery, starvation resistance, fitness, and lifespan. The genomes of the full suite of lines were fully sequenced using a combination of Illumina and 454 sequencing platforms. The sequencing identified polymorphisms, such as single nucleotide polymorphisms (SNPs) and microsatellites. With this resource, it is possible to measure traits of individual lines and carry out genome wide association (GWA) analyses to identify polymorphisms that are significantly associated with the tested phenotype.

Several studies have successfully utilized the DGRP to explore the genetic basis of the innate immune response. These studies have identified novel variants that affect response to oxidative stress, lifespan and fitness, as well as age and diet-related resistance to bacterial infections (Durham et al., 2014; Felix et al., 2012; Jordan et al., 2012; Magwire et al., 2012; Unckless et al., 2015). For example, a GWAS to identify polymorphisms that are associated with resistance to *Drosophila C Virus* (DCV) in the DGRP identified 6 highly significant SNPs in the gene *pastrel* (Magwire et al., 2012). This gene had not been previously associated with anti-viral immunity and it encodes a protein of unknown function. Flies expressing *pastrel*-RNAi ubiquitously are more

susceptible to DCV infection compared to control flies of the same genetic background. The role of *pastrel* as an important host factor for DCV infection was independently verified in a study that examined the genetics of fly adaptation to DCV (Martins et al., 2014; Martins et al., 2013). An outbred population of *Drosophila* was exposed to recurrent DCV infections over the course of several generations. Comparison of allele frequencies across those generations revealed five highly polymorphic loci, the most significant of which were associated with *pastrel.* Thus, two independent GWAS approaches successfully identified *pastrel* as a central player in the host response to DCV. Together, these findings demonstrate the utility of GWAS in *Drosophila* innate immune research. In conclusion, there is still much to discover about the genes and signaling pathways that regulate the cellular immune response to bacteria in *Drosophila.*

In an effort to identify new genes associated with phagocytosis and phagosome maturation in the fly, we carried out three separate GWAS studies: *S. aureus* phagosome maturation (to potentially identify genes that control uptake and maturation), *E. coli* phagocytosis, and *S. aureus* phagocytosis. Together, these studies identified over 500 candidate genes than may be important for the cellular immune response in *Drosophila.* We also carried out an in depth analysis of one *S. aureus* phagocytosis candidate gene, the RNA-binding protein Ataxin-2 binding-protein 1 (A2bp1).

B. Immunoselection and RNAseq to examine the adult hemocyte transcriptome

Several published microarray and mass spectrometry experiments have documented changes in the expression of a number of transcripts after bacterial infection in the fruit fly (Boutros et al., 2004; De Gregorio et al., 2001; De Gregorio et al., 2002; Handke et al., 2013; Irving et al., 2005; Levy et al., 2004; Uttenweiler-Joseph et al.,

1998). In general, gene expression changes have been examined using samples that are comprised of whole animals, potentially masking hemocyte-specific transcriptional changes. None of these previous studies has analyzed the changes in gene expression in adult hemocytes after infection. Genetic ablation studies have established that hemocytes synthesize humoral immune effector molecules in response to infection (Agaisse et al., 2005; Avet-Rochex et al., 2005; Braun et al., 1998b; Matova and Anderson, 2006). *Drosophila* hemocytes secrete cytokine-like molecules after bacterial infection. One cytokine, Upd3, is released into the hemolymph after the hemocyte senses *E. coli* or *M. luteus* (Agaisse et al., 2005). Upd3 is the ligand for the cytokine receptor, Domeless, which is found on fat body cells. Upd3 binds to Domeless, which triggers the initiation of the JAK/STAT signaling cascade, leading to the production of stress genes such as *TurandotA*. Expression of the Toll ligand, Spatzle is also increased in hemocytes after infection (Irving et al., 2005). In larvae, genetic ablation of hemocytes impairs the expression of the AMP genes *Diptericin* and *Drosomycin* from the fat body (Shia et al., 2009). Blood cell-specific *spz*-RNAi also led to impaired AMP expression but this defect was not observed when *spz* was silenced specifically in the fat body. Finally, hemocyte-specific expression of wild type *spz* in a *spz*-null background is sufficient to rescue the impaired AMP induction. These findings indicate that the hemocytes secrete Spatzle after sensing bacteria, and this secretion is necessary for Toll-dependent AMP production by the fat body. However, the existence of cross-talk between hemocytes and the fat body is a subject of debate in the literature. In adults, genetic ablation of blood cells does not appear to affect the induction of AMP gene expression after Gram-positive or Gram-negative bacterial infections (Charroux and Royet, 2009; Defaye et al., 2009).

Furthermore, rendering hemocytes unable to phagocytose bacteria by pre-injecting flies with latex beads also did not impair the immune response in the same flies after bacteria infection (Nehme et al., 2011).

Based on the work outlined above, several questions remain: Do adult or larval hemocytes secrete effectors that induce the humoral immune response in fat body cells? Does gene expression robustly change in hemocytes after recognition and phagocytosis of microbes? If so, how do different pathogens differentially affect gene expression in blood cells? Overall, the transcriptional response of adult *Drosophila* hemocytes following microbial challenge has been relatively unexplored. Using immunoselection and RNAseq, we generated a list of genes that are differentially expressed in adult hemocytes after wounding and after *S. aureus* or *Drosophila X Virus* infection. Our list may provide the foundation for future studies that elucidate the role of hemocyte-specific gene expression changes in the immune response in the fly. In conclusion, there is still much to discover about the genes and signaling pathways that regulate the cellular immune response to bacteria in *Drosophila.* In an effort to identify new genes associated with phagocytosis and phagosome maturation in the fly, we carried out three separate GWAS studies: *S. aureus* phagosome maturation (to potentially identify genes that control uptake and maturation), *S. aureus* phagocytosis, and *E. coli* phagocytosis. Furthermore, the transcriptional response of adult *Drosophila* hemocytes following microbial challenge has been relatively unexplored. Using immunoselection and RNAseq, we generated a list of genes that are differentially expressed in adult hemocytes after wounding and after *S. aureus* or *Drosophila X Virus* infection.

Chapter 2

Genome Wide Association Analysis in a Natural Population of *Drosophila* Reveals Novel Mediators of the Cellular Response to *Staphylococcus aureus*

I. Abstract

In all animals, the innate immune response is the first line of defense against microorganisms. Phagocytosis, the recognition and uptake of particles greater than 0.5 μm, is a vital component of innate immunity and is particularly important for protection against the Gram-positive bacterium *Staphylococcus aureus*. *S. aureus*, which is often found as a commensal in human flora, causes a multitude of illnesses in humans if left unchecked. To identify genes affecting phagosome maturation *in vivo*, we tested a subset of the Drosophila Genetic Reference Panel (DGRP) for the ability of their hemocytes (phagocytic blood cells) to uptake and degrade *S. aureus*. The phagosome maturation phenotypes were sexually dimorphic and highly variable within individual lines. We then performed genome-wide association analyses to identify candidate genes associated with male, female, and pooled phagosome maturation phenotypes. We identified potentially relevant single nucleotide polymorphisms (SNPs) near or in hundreds of genes, many of which have no known role in the cellular immune response to bacteria. Gene Ontology analyses revealed that our list was enriched for plasma membrane associated proteins and proteins with Ig-like motifs. Thirty-eight candidate genes were selected for further analysis and sex-specific effects on phagosome maturation were assessed using RNA interference in hemocytes. We identified three genes, *dpr10, fred,* and *CG42673,* whose

loss-of-function in blood cells impaired the innate immune response to *S. aureus*. Finally, many of the candidate genes identified in the screen have human orthologs. Owing to the high degree of genetic conservation between humans and Drosophila, our findings may facilitate future studies that could shed light on the human innate immune response to *S. aureus*.

II. Introduction

The cellular immune response is an immediate and crucial component of host defense. Recognition and clearance of invasive bacteria by phagocytic cells is essential for an effective immune response. Phagocytes target two main classes of molecules: apoptotic cells and microorganisms. Throughout development, removal of apoptotic cells is key for tissue remodeling. However, during the innate immune response, phagocytic cells recognize, engulf and kill invading microbes (Akira et al., 2006; Blander and Sander, 2012; Flannagan et al., 2012; Jiravanichpaisal et al., 2006; Stuart and Ezekowitz, 2008). Phagocytosis is particularly vital for protection against extracellular bacteria, such as the Gram-positive bacterium *Staphylococcus aureus (S. aureus*). *S. aureus* can live as a commensal in human flora, where it is controlled by neutrophils and macrophages (Ip et al., 2010; Miller and Cho, 2011; Spaan et al., 2013). Defects in these cells can lead to severe immunodeficiency resulting in recurring and persistent bacterial infections. If left unchecked, *S. aureus* can cause a myriad of illnesses such as skin infections, respiratory disease, toxic shock, bacteremia, and sepsis (van Kessel et al., 2014).

Drosophila melanogaster is a genetically tractable organism well suited for studying the cellular immune response. Many of the genes and signaling pathways of innate immunity are conserved between fruit flies and humans. This genetic conservation, coupled with the ability to rapidly phenotype a large number of individuals, makes Drosophila a powerful model for the study of innate immunity. (Buchon et al., 2014; Cherry and Silverman, 2006; Dionne and Schneider, 2008; Ferrandon et al., 2007; Ganesan et al., 2011; Lemaitre and Hoffmann, 2007).

The fruit fly lacks an adaptive immune response, but possesses a multifaceted and highly effective innate immune response that can be divided into two branches: the humoral and cellular responses. Humoral immunity is a systemic response mediated mainly by the fat body, a functional equivalent of the mammalian liver. Upon infection, immune responsive genes such as Turandots, Teps and antimicrobial peptides (AMPs), are transcriptionally activated via the Toll and Imd pathways. The Toll pathway is triggered by detection of fungi, Drosophila X Virus, and Lys-type peptidoglycan of Gram-positive bacteria (Lemaitre et al., 1996; Leulier et al., 2003; Michel et al., 2001a; Zambon et al., 2005). Toll signaling results in the induction of AMP genes, such as *Drosomycin,* following the nuclear translocation of dorsal-related immune factor (DIF) (Ip et al., 1993; Lemaitre et al., 1996). The Imd pathway is activated by detection of DAP-type peptidoglycan of Gram-negative bacteria, leading to the nuclear translocation of the NF-κB-like transcription factor, Relish, which upregulates the transcription of AMP genes such as *Diptericin*. (Hedengren et al., 1999; Kaneko et al., 2004; Lemaitre et al., 1995; Leulier et al., 2003; Leulier et al., 2000).

Insect hemocytes, blood cells that are the functional equivalent of the mammalian macrophage, mediate the cellular immune responses of phagocytosis, nodulation, encapsulation and melanization (Fauvarque and Williams, 2011; Jiravanichpaisal et al., 2006; Meister and Lagueux, 2003). Phagocytosis is initiated when pattern recognition receptors (PRRs) on the surface of phagocytic cells identify and bind to pathogen associated molecular patterns (PAMPs) on the surface of microbes. Bound receptors activate signaling pathways that lead to internalization of particles in an actin-dependent manner. The organelle that forms around the engulfed material, the phagosome,

undergoes the process of phagosome maturation, a series of fusion events with endosomes and lysosomes that results in a highly acidic and hydrolytic phagolysosome where antimicrobial proteins and peptides digest microbes. Phagocytosis and phagosome maturation are reviewed in (Flannagan et al., 2012; Kinchen and Ravichandran, 2008; Sarantis and Grinstein, 2012; Swanson, 2008; Underhill and Goodridge, 2012).

Several studies have demonstrated that humoral and cellular immune responses work together to combat bacterial infections in the fruit fly (Braun et al., 1998a; Brennan et al., 2007; Matova and Anderson, 2006; Nehme et al., 2011; Pham et al., 2007). In one study, Imd mutant flies succumbed to an otherwise harmless Gram-negative *Escherichia coli* infection only when the cellular immune response is ablated by pre-injection with latex beads (Elrod-Erickson et al., 2000). Alternatively, loss of *Psidin,* a lysosomal protein, impairs phagosome maturation of Gram-positive and Gram-negative bacteria in hemocytes, but also leads to deficient induction of the AMP gene *Defensin* in the fat body (Brennan et al., 2007). This type of crosstalk is critical for an effective host response against a multitude of bacterial pathogens. However, experiments in fruit flies as well as in another insect model, the beetle *Tenebrio molitor*, indicate that hemocyte-mediated defenses, and not the humoral immune response, are the most immediate and critical component of the immune response to *S. aureus* (Haine et al., 2008; Nehme et al., 2011). In *Tenebrio molitor*, hemocytes effectively clear 99.5% of *S. aureus* within an hour of infection. The humoral immune response is induced after the majority of the bacteria are eliminated by hemocytes, and AMPs control the growth and dissemination of remaining pathogens (Haine et al., 2008). Thus, in both insects and humans, blood cell recognition

and lysosomal degradation of internalized microbes is essential to combat *Staphylococcus aureus* infections.

Phagocytosis processes and post-engulfment events are evolutionarily conserved: several characterized fruit fly and human germline encoded pattern recognition receptors are homologous and pathogen recognition leads to activation of similar signaling cascades(Lemaitre and Hoffmann, 2007; Stuart and Ezekowitz, 2005). Biochemical and genetic analyses in Drosophila have yielded valuable insights into the biological complexity of phagocytosis. Cell-based RNA interference in the macrophage-like S2 cell line and classical genetic screening strategies have successfully identified a number of Drosophila phagocytic receptors (reviewed in (Marmaras and Lampropoulou, 2009; Ulvila et al., 2011)) including: the CD36 family scavenger receptors Peste (Philips et al., 2005) and Croquemort (Stuart et al., 2005), Scavenger receptor class C, type I (Sr-CI) (Ramet et al., 2001), peptidoglycan recognition protein LC (PGRP-LC) (Ramet et al., 2002), the Ig-like protein Down syndrome cell adhesion molecule (Dscam) (Watson et al., 2005), and three EGF-like repeats containing proteins Nimrod C1 (Kurucz et al., 2007a), Eater (Kocks et al., 2005), and Draper (Cuttell et al., 2008; Manaka et al., 2004). The binding specificities of Drosophila phagocytic receptors often overlap, suggesting a level of redundancy that is similar to mammalian cells. Because multiple receptors target common ligands, knockdown of individual receptors and downstream signaling molecules may not lead to complete loss of phagocytic capacity. Additionally, screens that target single genes using loss-of-function or gain-of-function mutants may not identify novel candidates with subtle effects or genes that have epistatic effects on one another.

In this study, we undertook a complementary approach to mutagenesis screens with the aim of identifying novel genes that play a role in the cellular immune response to *S. aureus*. We conducted a genome wide association study (GWAS) using a fully sequenced panel of inbred lines, the Drosophila Genetic Reference Panel (DGRP) (Mackay et al., 2012) (Figure 2-1). The DGRP has been utilized to identify novel variants that affect viral immunity, response to oxidative stress, lifespan and fitness, as well as age and diet-related resistance to bacterial infections (Durham et al., 2014; Felix et al., 2012; Jordan et al., 2012; Magwire et al., 2012; Unckless et al., 2015). Alleles with more subtle effects segregate in natural populations such as the DGRP (Mackay et al., 2012). We hypothesized that a GWA analysis of DGRP lines using an *in vivo S. aureus* phagosome maturation assay would identify novel polymorphisms with subtle and synergistic effects.

We tested DGRP lines for the ability of their hemocytes to phagocytose and degrade *S. aureus* bioparticles labeled with a pH-sensitive fluorochrome and we performed GWA analyses for the median phagosome maturation phenotypes using a mixed effect analysis of variance (ANOVA) model. At *p*-values less than $1x10^{-4}$ we identified 985 single nucleotide polymorphisms (SNPs) near (< 5000 bp away from) or within 528 genes. Eighty-two candidate genes are predicted to localize to the plasma membrane and 21 encode proteins with immunoglobulin-like domains. Several SNPs were located in or near genes that play a role in the Drosophila innate immune response, cytoskeletal organization, vesicle trafficking or lysosomal function. Twenty-seven of the identified genes have human orthologs implicated in the immune response.

We then performed follow-up studies to assess the role of candidate genes of interest, including 15 genes encoding proteins that localize to the plasma membrane and 12 genes that encode proteins with Immunoglobulin-like domains. We reasoned that focusing on proteins potentially found at the periphery of the hemocyte might facilitate the identification of novel pathogen recognition receptors or downstream signaling molecules that mediate internalization of *S. aureus*. We selected forty candidate genes, used RNA-interference to silence expression specifically in hemocytes, and screened adults using the *in vivo* phagosome maturation assay (Duffy, 2002; Ni et al., 2008). RNAi knockdown of three candidates: *defective proboscis extension response 10 (dpr10)*, *friend of echinoid* (*fred*), and *CG42673* significantly impaired *S. aureus* phagosome maturation. This cellular immune defect also led to increased susceptibility to *S. aureus* infection. Susceptibility to bacterial infection may be attributed to decreased resistance (inability to curb bacterial growth) or decreased tolerance (inability to mitigate the harmful effects of the immune response). Quantification of *S. aureus* bacteria load 24 and 48 hours post infection revealed that loss of candidate genes in hemocytes led to changes in resistance and tolerance in a sex specific manner. Thus, using a combined approach of GWAS and RNA interference to silence candidate genes in hemocytes, we have identified novel regulators of *S. aureus* phagocytosis.

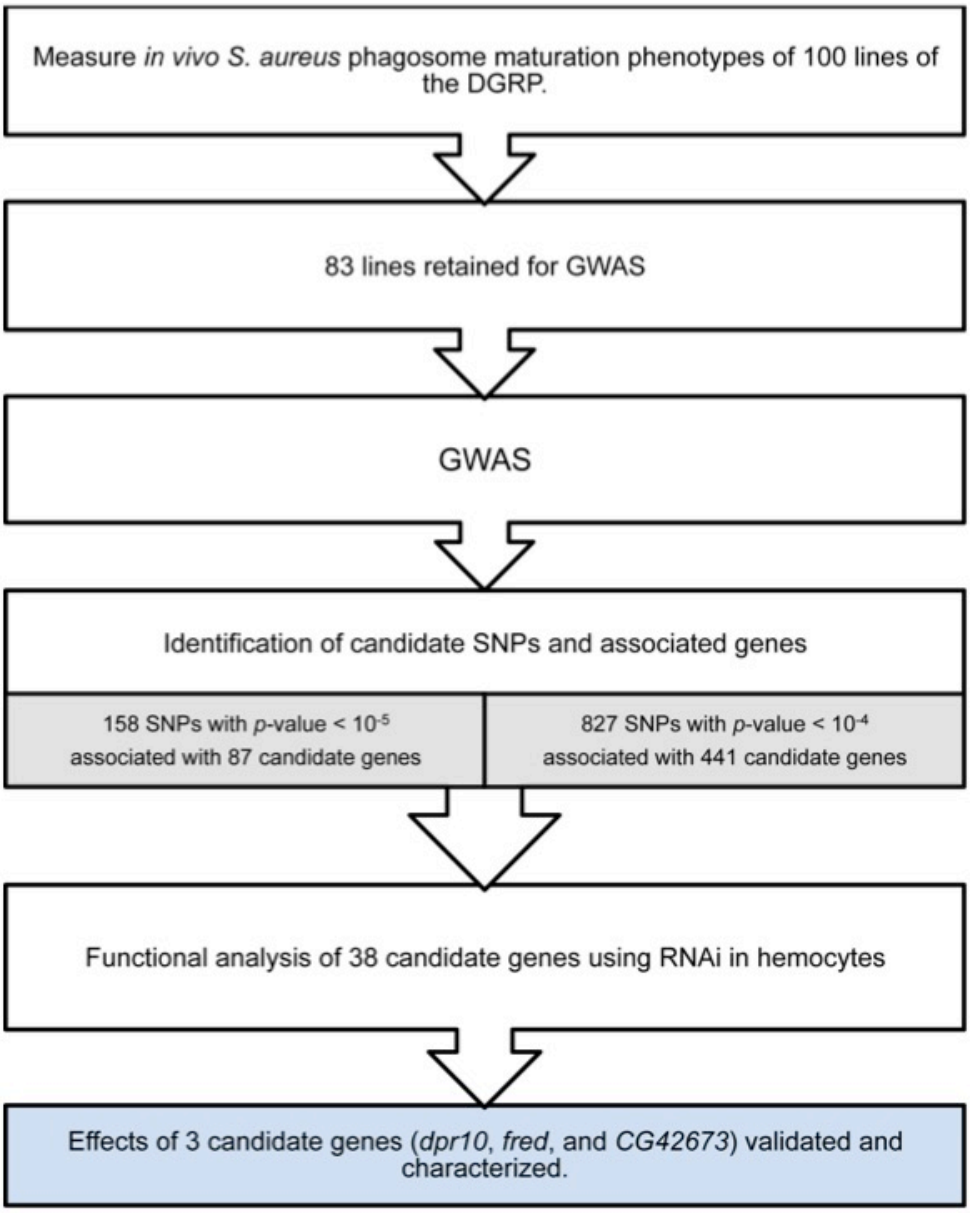

Figure 2-1: Overview of the *S. aureus* phagosome maturation screen.

The flow-chart is a summary of the design and results of the genome-wide association study to identify single nucleotide polymorphisms and genes that are associated with *S. aureus* phagocytosis and phagosome maturation in the DGRP.

III. Results

A. An *in vivo* phagosome maturation screen

To identify novel genes or signaling pathways that regulate *S. aureus* phagocytosis and phagosome maturation, adult DGRP flies were assessed for the ability of their hemocytes to phagocytose pHrodo-conjugated *S. aureus*. The pHrodo dye is pH-sensitive: it is non-fluorescent at neutral pH but is bright red in acidic compartments with low pH. Following uptake by hemocytes, the microbe-containing phagosome matures through a series of fusion events with increasingly acidic endosomal compartments. The pHrodo-conjugated *S. aureus* will fluoresce brightly as maturation progresses. Sessile hemocytes along the dorsal vessel phagocytose dye-conjugated microbes and can be visualized through the cuticle (Figure 2-2) (Elrod-Erickson et al., 2000). The absence of pHrodo fluorescence could be attributed to defects in particle uptake (phagocytosis) or to defects in trafficking and/or acidification of the phagosome (phagosome maturation). Thus, use of pHrodo-conjugated *S. aureus* provided a way to simultaneously assess phagocytosis and downstream maturation processes in the DGRP lines.

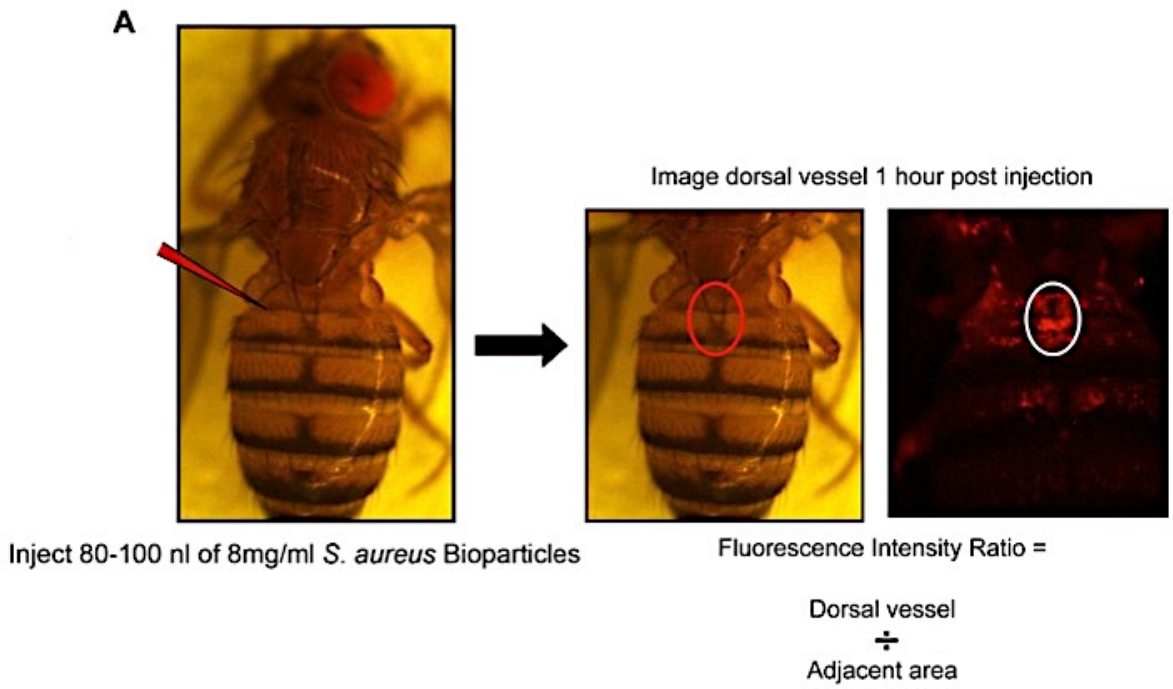

Figure 2-2: Schematic of the in vivo phagosome maturation assay.

Adult male and female flies were injected with heat-killed *S. aureus* labeled with pHrodo, a fluorescent dye that only fluoresces at low pH (~pH 4.5). Flies were allowed to rest one hour to allow the hemocytes time for the steps of phagocytosis and phagosome maturation to occur: i.e. particle recognition by hemocytes, particle uptake, formation of the phagosome, and maturation of *S. aureus*-pHrodo containing phagosomes. After 1 hour, flies were mounted on electrical tape and the dorsal vessel, an area where a large number of sessile hemocytes congregate, was images on in inverted fluorescence microscope. The fluorescence intensity of the dorsal vessel and an adjacent, non-fluorescent area on the fly cuticle, was manual measured for each fly. The fluorescence intensity ratio of each fly was calculated by normalizing the fluorescence of the dorsal vessel by the fluorescence of the adjacent area.

Seven males and seven females were tested from 100 randomly selected DGRP lines. We also included an isogenic laboratory strain, *cnbw*, to control for experimental variability. The fluorescence intensity ratio was calculated for each fly, and median phagosome maturation phenotypic value was determined per line (Table 2-1 and Figure 2-3). Lines for which the phenotypic variability exceeded a standard deviation of 0.5 were removed from the GWA analysis. An additional 5 lines were eliminated, as they were no longer supported by the DGRP Bioinformatics pipeline (Table 2-1 and Figure 2-3). We utilized the remaining 83 lines for all further analysis. Finally, we normalized the raw phenotypic data of each line by dividing DGRP phagosome maturation phenotypes by the *cnbw* phagosome maturation value for the date tested (Figure 2-4). Phagosome maturation in the DGRP was sexually dimorphic: the average value for males (0.8534) was significantly lower than for females (1.004) ($p < 0.0001$) (Figure 2-4 and 2-5). There was considerable genetic variation in the sexual dimorphism, due to the fact that differences in phagosome maturation between males and females varied among lines (Figure 2-5). This observation is consistent with observations in other immune and complex trait studies (Harbison et al., 2013; Spitzer, 1999; Taylor and Kimbrell, 2014).

Table 2-1: Summary of the DGRP *S. aureus* phagosome maturation screen.

DGRP Strain	Phagosome Maturation AVERAGE / MEDIAN	# of flies tested	Standard Deviation	Included in GWA
cnbw	1.579 / 1.535	78	0.3604	(-) Laboratory control
argus	1.072 / 1.070	32	0.1480	(-) Laboratory control
21	1.523 / 1.335	13	0.5023	(-) STD > 0.5
26	1.609 / 1.585	14	0.3025	+
38	1.486 / 1.502	14	0.2170	+
40	1.719 / 1.664	14	0.3675	+
41	1.227 / 1.178	14	0.2357	+
42	1.665 / 1.694	14	0.4842	+
45	1.403 / 1.357	14	0.3471	+
57	1.812 / 1.636	14	0.7516	(-) STD > 0.5
59	1.635 / 1.536	14	0.4295	+
69	1.491 / 1.416	13	0.2829	+
73	1.197 / 1.171	14	0.1309	+
75	1.240 / 1.203	14	0.2421	+
83	1.197 / 1.095	14	0.2416	+
85	1.674 / 1.567	14	0.3611	+
91	1.689 / 1.721	13	0.4201	+
93	1.158 / 1.159	13	0.1385	+
101	1.675 / 1.626	14	0.3251	+
105	1.699 / 1.626	15	0.4618	+
109	1.368 / 1.322	14	0.3421	+
129	1.579 / 1.565	14	0.5032	(-) STD > 0.5
136	1.519 / 1.555	13	0.2393	+
138	1.389 / 1.298	14	0.2472	+
142	1.416 / 1.361	14	0.3394	+
149	1.250 / 1.116	14	0.3036	+
158	1.421 / 1.463	14	0.2368	+
161	1.483 / 1.463	13	0.3453	+
176	1.478 / 1.442	14	0.3362	+
177	1.303 / 1.270	14	0.2670	+
195	1.647 / 1.490	14	0.4759	+
208	1.741 / 1.567	15	0.3832	+
217	1.649 / 1.286	13	0.6016	(-) STD > 0.5
223	1.277 / 1.313	14	0.1562	(-) No longer curated by DGRP
227	1.479 / 1.348	13	0.4401	+
228	1.551 / 1.496	14	0.2177	+
235	1.438 / 1.497	15	0.3927	+
239	1.441 / 1.346	13	0.4558	+
280	1.406 / 1.272	15	0.4880	+
301	1.208 / 1.159	14	0.2305	+
303	1.504 / 1.379	14	0.3035	+

304	1.415 / 1.424	14	0.2702	+
306	1.619 / 1.466	15	0.6536	(-) STD > 0.5
307	1.264 / 1.228	14	0.2226	+
309	1.829 / 1.925	14	0.6109	(-) STD > 0.5
310	1.427 / 1.309	15	0.4957	+
313	1.340 / 1.367	14	0.2234	+
315	1.538 / 1.386	14	0.4527	+
317	1.594 / 1.526	14	0.3491	+
318	1.727 / 1.675	14	0.4444	+
320	1.565 / 1.331	14	0.5965	(-) STD > 0.5
324	1.430 / 1.403	13	0.2668	+
335	1.652 / 1.619	15	0.29	+
340	1.633 / 1.564	14	0.4583	(-) No longer curated by DGRP
350	1.612 / 1.471	14	0.6263	(-) STD > 0.5
357	1.469 / 1.414	12	0.2860	+
358	1.274 / 1.331	14	0.1607	+
360	1.617 / 1.703	14	0.3082	+
362	1.390 / 1.277	13	0.3088	+
365	1.521 / 1.497	14	0.3687	+
370	1.837 / 1.710	13	0.4291	+
373	1.614 / 1.419	14	0.5861	(-) STD > 0.5
374	1.341 / 1.360	14	0.2548	+
375	1.656 / 1.617	14	0.2226	+
379	1.895 / 1.693	13	0.5005	(-) STD > 0.5
380	1.427 / 1.387	13	0.3306	+
392	1.206 / 1.163	14	0.2465	+
399	1.547 / 1.496	14	0.2415	+
427	1.364 / 1.309	14	0.2193	+
437	1.307 / 1.300	13	0.1940	+
439	1.443 / 1.357	14	0.3320	+
440	2.034 / 1.907	14	0.5867	(-) STD > 0.5
441	1.196 / 1.058	12	0.4310	+
486	1.584 / 1.499	14	0.3158	+
508	1.405 / 1.234	14	0.3718	+
514	1.889 / 1.895	14	0.5122	(-) STD > 0.5
517	1.829 / 1.851	12	0.3761	+
555	1.504 / 1.406	14	0.2384	+
639	1.376 / 1.353	13	0.2928	+
707	1.386 / 1.412	13	0.1980	+
712	1.557 / 1.406	14	0.2513	+
714	1.397 / 1.323	13	0.2120	+
705	1.474 / 1.366	14	0.4239	+
730	1.406 / 1.393	13	0.2070	+
732	1.467 / 1.502	14	0.2477	+
761	1.434 / 1.386	15	0.4297	+
765	1.694 / 1.546	13	0.4048	+

774	1.597 / 1.581	14	0.3557	+
787	1.611 / 1.597	15	0.2544	+
799	1.689 / 1.740	13	0.3610	+
804	1.798 / 1.849	14	0.5627	(-) STD > 0.5
810	1.780 / 1.760	14	0.4562	+
820	1.340 / 1.306	14	0.2591	+
822	1.742 / 1.835	13	0.2891	+
843	1.650 / 1.742	15	0.3276	(-) No longer curated by DGRP
852	1.675 / 1.680	15	0.3389	+
859	1.843 / 1.742	14	0.5773	(-) STD > 0.5
884	1.595 / 1.601	13	0.2842	+
892	1.466 / 1.395	13	0.1912	+
900	1.623 / 1.449	12	0.4101	(-) No longer curated by DGRP
911	1.282 / 1.218	12	0.3090	+

Table 2-1: Summary of DGRP S. aureus phagosome maturation screen raw data.

One hundred randomly selected DGRP strains were tested for the ability of their hemocytes to phagocytose and eliminate pHrodo-labeled, heat-killed *S. aureus* bioparticles. Three to 5 day old male and female flies were tested (n =12-15 flies per strain). Median phagosome maturation for each strain is given in column 2. Standard deviation within each strain was calculated and lines were removed from the GWA analysis if STD was > 0.5 or if the line was no longer curated by the DGRP bioinformatics pipeline.

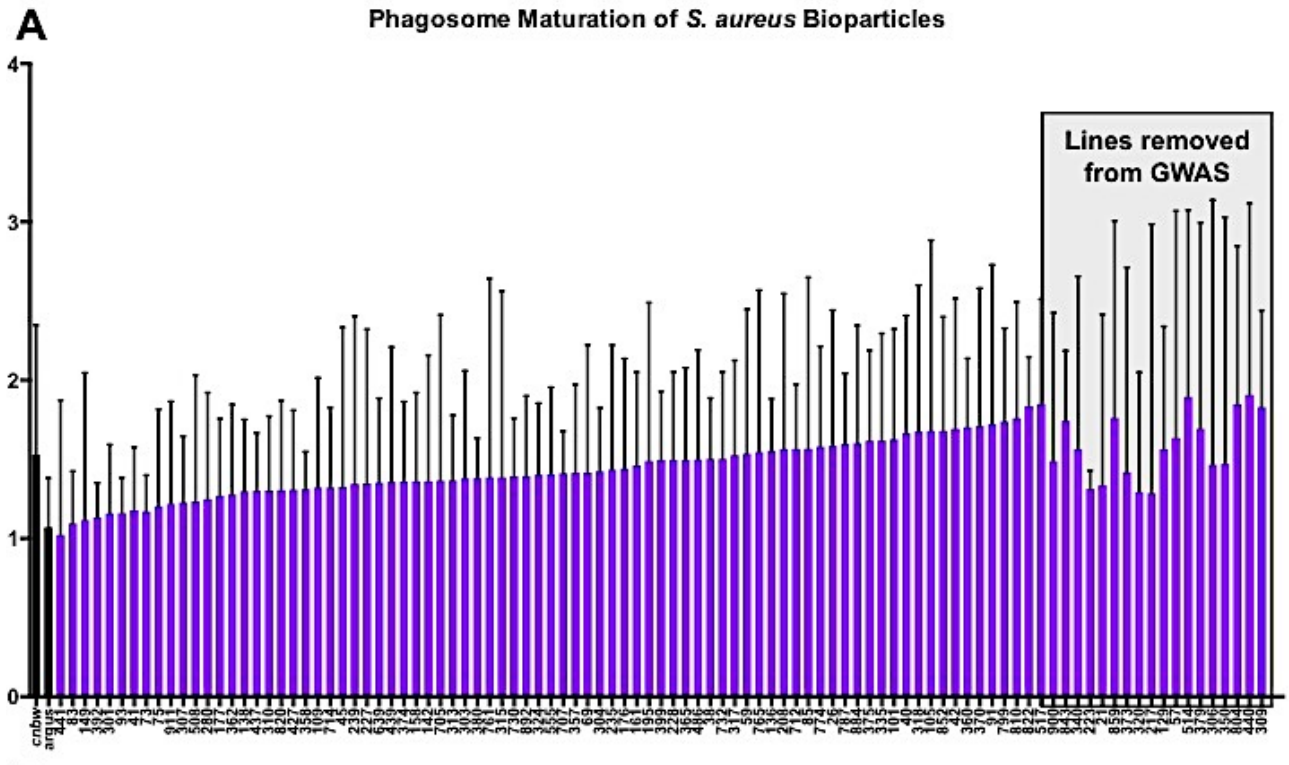

Figure 2-3: S. aureus phagosome maturation phenotypes of 100 DGRP lines.

DGRP line means for all 100 tested lines are shown. The shaded grey box indicates the lines that were removed from the GWA analysis (See Table 1-1 for further explanation)

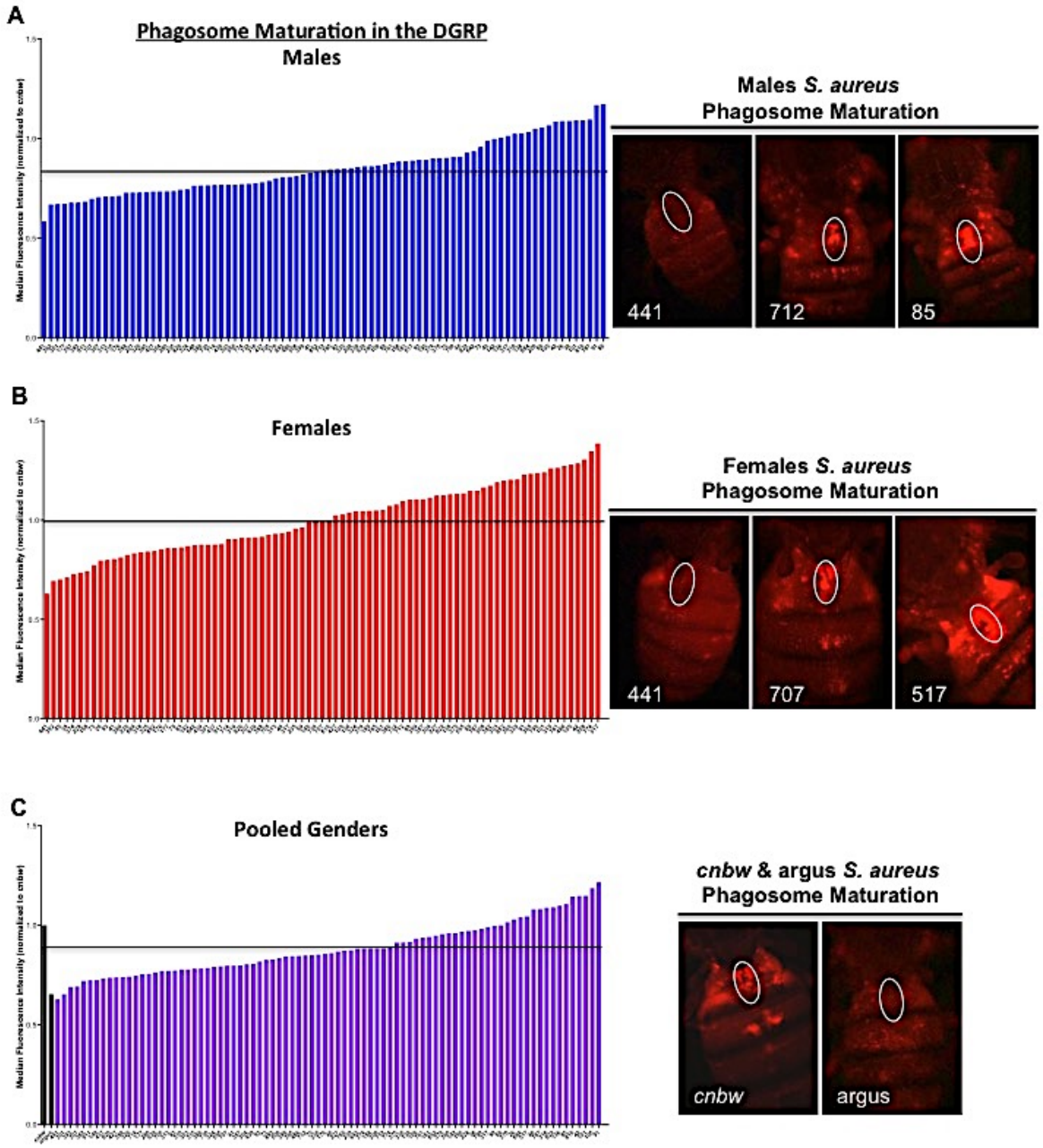

Figure 2-4: *S. aureus* phagosome maturation phenotypes in 83 DGRP lines.

The median pHrodo-*S. aureus* fluorescence intensity for each DGRP line is shown for (A) males, (B) females, and (C) pooled sexes. The mean across all lines is indicated with a black line. Representative images of pHrodo-fluorescing hemocytes from controls and DGRP lines with reduced, average, or increased *S. aureus* phagosome maturation are shown.

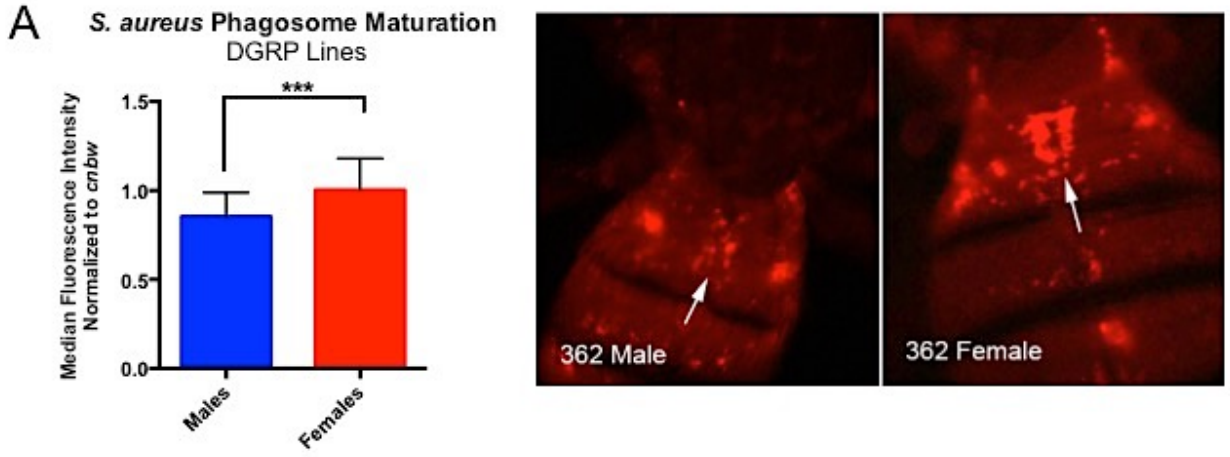

Figure 2-5: Sexual dimorphism in *S. aureus* phagosome maturation in the DGRP.

(A) Bar graph representation the average phagosome maturation value for DGRP males (0.8534) and females (1.004) ($p < 0.0001$). Representative images of *S. aureus* phagosome maturation in male (left) and female (right) flies.

B. Genome-wide association mapping

The genomes of all the DGRP lines have been sequenced, enabling us to carry out genome-wide genotype-phenotype association mapping for *S. aureus* phagosome maturation phenotypes (Mackay et al., 2012). The DGRP analysis pipeline (http://dgrp2.gnets.ncsu.edu) utilizes Flybase release 5.49 to annotate 2.49 million single nucleotide polymorphisms (SNPs) in the DGRP for which the minor allele is present in at least 4 lines. To identify SNPs significantly associated with our phenotypic measurements, we performed GWA analyses for the median phagosome maturation values of males and females using the DGRP analysis pipeline (Mackay et al., 2012).

As suggested by Mackay *et al*., we adopted a *p*-value threshold of less than 10^{-5} for our initial GWA analysis. At this significance threshold, we anticipate a false discovery rate (FDR) of 0.6 (see methods). In an effort to uncover additional genes or signaling pathways that might play a role in *S. aureus* phagosome maturation in Drosophila, we subsequently relaxed our threshold to include SNPs with *p*-values < $10^{-4,}$ anticipating a false discovery rate of 0.7. We tolerated these lenient false positive rates due to the fact that we can test the functional significance of candidate genes within hemocytes *in vivo.*

At a significance threshold of *p*-value < $1x10^{-5}$ we identified 34 SNPs within or near (< 5000 bp away from) 27 candidate genes in males and 41 SNPs in or near 19 candidate genes in females (Table 2-2 and Online Supplemental Table 1). There was no overlap between the genes identified in the male GWA compared to the female GWA, providing further evidence that genetic factors contribute to the sexually dimorphic phenotypic difference between the sexes. To identify SNPs with similar effects in both

males and females, we ran a GWA analysis for the median phagosome maturation phenotype from each individual line (pooled across sexes). We found 36 SNPs (p-value < 10^{-5}) in or near 20 genes associated with the pooled sex phenotypes (Table 1-2 and Online Supplemental Table 1). Interestingly, 6 SNPs were found in both males and pooled GWA analysis, revealing that the male phenotypic measurements dominated the screen. For each associated SNP with p-value < 10^{-5} the following GWA analysis results were obtained from the Mackay lab bioinformatics analysis: p-values, identities of minor and major alleles, minor allele frequency (MAF) and estimates of effect size (calculated as the average difference in trait mean between lines carrying the major and minor alleles). Additionally, analyses of linkage disequilibrium among significant markers were reported (Fig 2-6). To visualize loci with multiple significant SNPs on a genome-wide scale, Manhattan plots of the negative $\log_{10}p$-value of the 2.5 million SNPs of the DGRP were generated (Fig 2-7). Quantile-quantile (Q-Q) plots of our GWAS results show that our observed p-values do not deviate significantly from the expected p-values for a screen of this size (Fig 2-7).

Gene ontology enrichment category	Median		
	# SNPs	# Genes	Associated genes
Males	34	27	*ATP7, stet, CR34701, snoRNA:Psi28S-2622, CG11498, Ca-beta, CG14280, CG42694, CG5987, CG5984, CR43838, shakB, PPYR1, CR43960, CG1950, CG6145, bbc, cdc14, Obp58c, Obp58d, Obp58b, CG15803, nrv1, CG32791, CG30096, CG8060, Ser*
Female	41	19	*CG42673, Dl, loco, mRpL45, Shab, Dhc16F, CG7536, dik, Ndae1, CG34351, Sh, rdx, Btd, chas, spri, CG5191, CG31952, pgant2, CG15161*
Pooled	36	22	*bbc, CG42732, CG11498, elk, kst, CG10732, CG10133, Egfr, CG13285, Ser, stet, CR43701, snoRNAi:Psi28S-2622, X11Lbeta, unc-104, Sod2, Pde1c, CanA-14F, Pkc98E, CG3264, lmd, stau*
Antagonistic	47	30	*bif, fas, CG18368, yps, dpr8, Xrp1, lr68b, Pvf3, CG34201, dpr, SNF4gamma, CaBP1, CG4455, CG17364, Dhc16F, CG6983, GluRIB, Ror, CG5676, CG45002, goe, CG32572, Roc2, Tret1-1, Sh, ncm, CG12782, Ndae1, loco, cdc14*

Table 2-2: Results of the genome-wide association of *S. aureus* phagosome maturation and SNPs at *p*-value<10^{-5}

GWAS results for median *S. aureus* phagosome maturation from 83 lines of the DGRP (s.d.<0.5). Median phagosome maturation is the middle value for each sex (male or female) or individual DGRP lines (pooled). Antagonistic SNPs were associated with significant differences between the sexes ($female_{phagosome\ maturation} - male_{phagosome\ maturation}$). The number of significant SNPs (p-value<10^{-5}) and associated genes are given. Gene names are given are listed in order of increasing p-value.

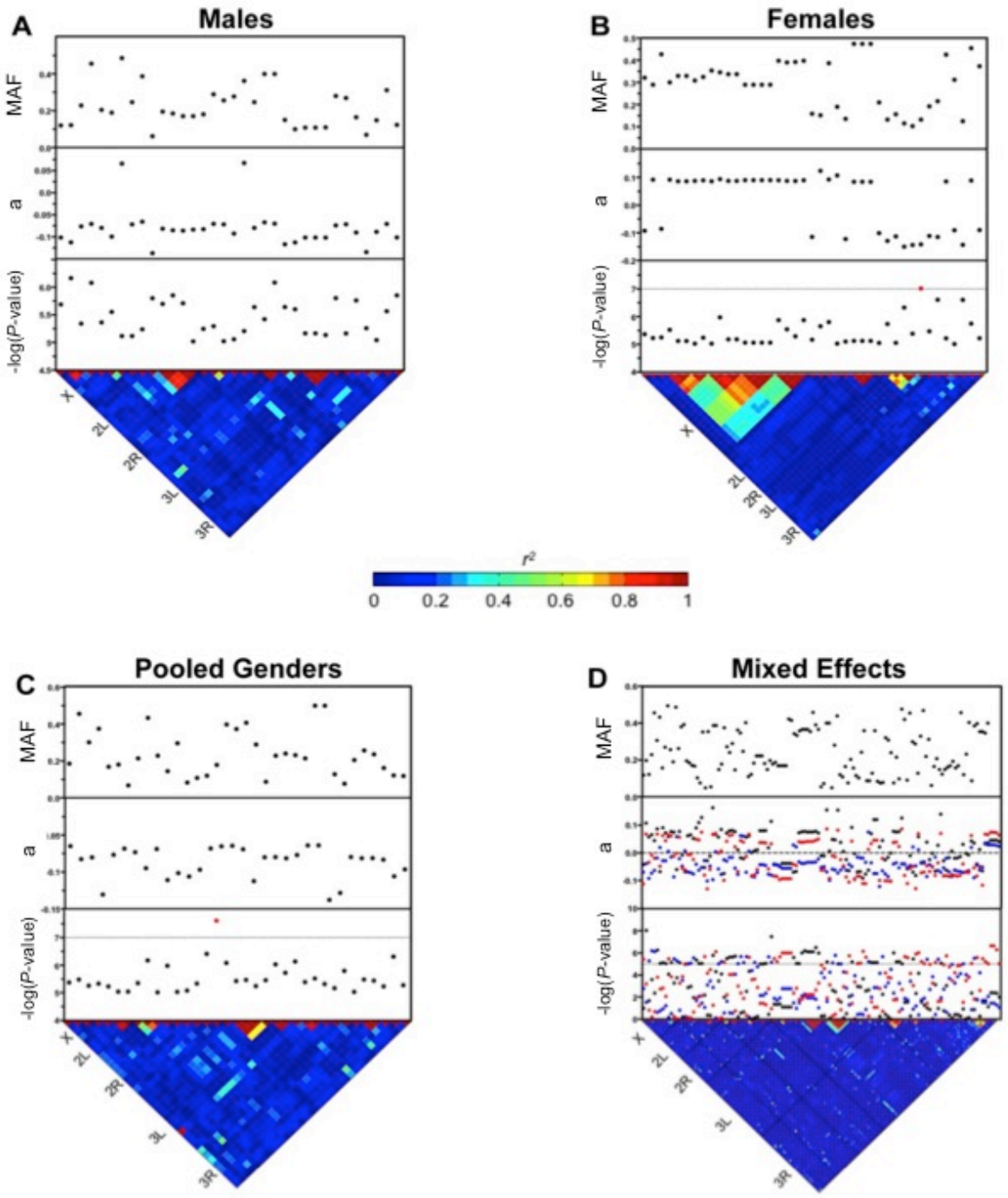

Figure 2-6: Genome-wide association analysis for *S. aureus* phagosome maturation.

Figure 2-6: Genome-wide association analysis for *S. aureus* phagosome maturation. All SNPs with minimum *p*-values < $1x10^{-5}$ from single-phenotype GWA for (A) males, (B) females, and (C) pooled sexes are shown. (D) All SNPs with minimum *p*-values < $1x10^{-5}$ from two-sex GWA to identify SNPs that exert antagonistic effects in males and females are shown. For the antagonistic SNPs red dots represent females, blue dots represent males, and black dots represent the difference between females and males. The lower triangle illustrates the degree of linkage disequilibrium (LD) between SNPs, measured by r^2. The R^2 heatmap indicates the degree of LD: red corresponds to high degrees of LD and blue to the absence of LD. The upper panels show the significance value ($-\log_{10}$*p*-value), the effect size (a), and the minor allele frequency (MAF) of each significant variant.

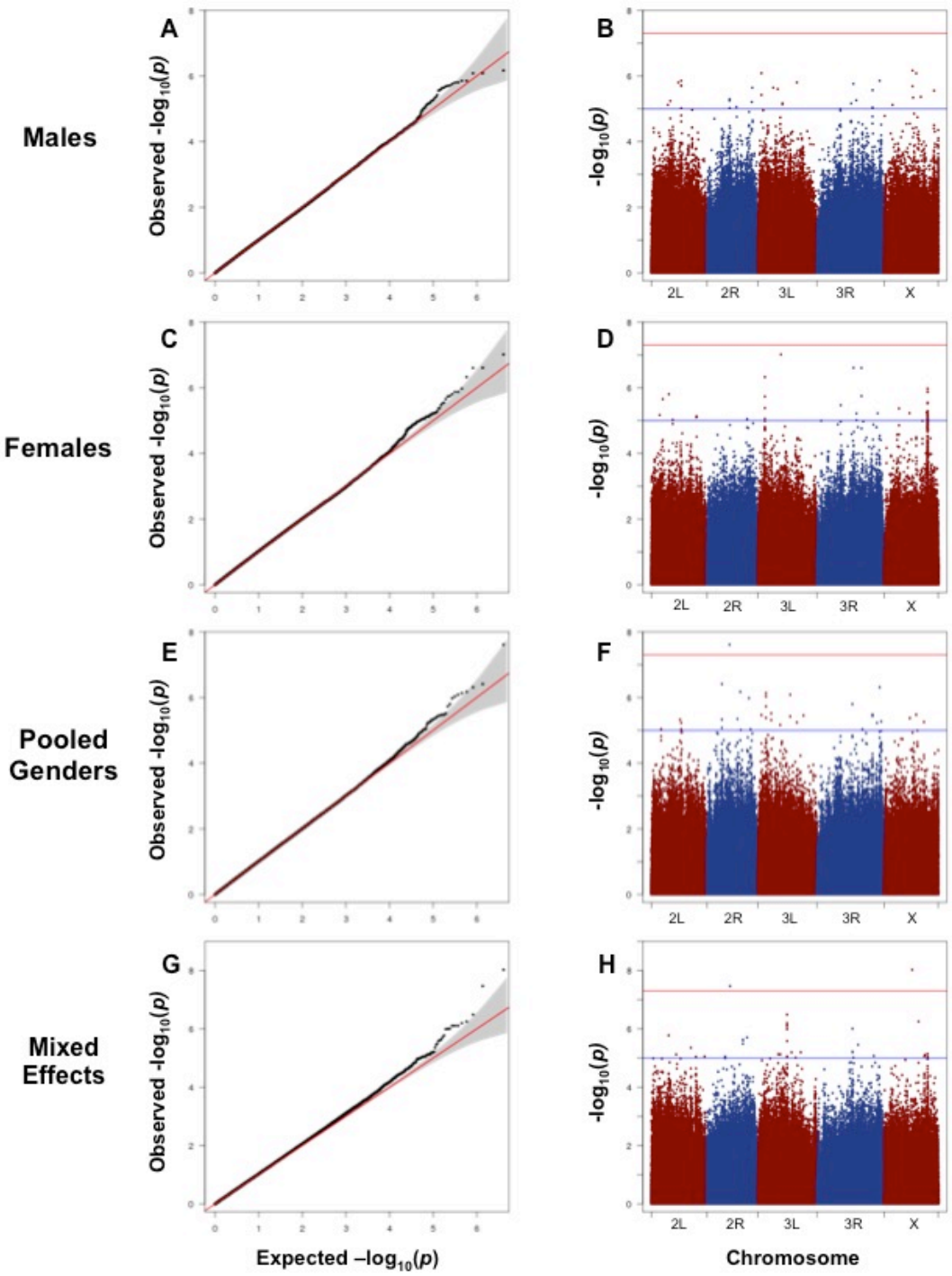

Figure 2-7: Quantile-Quantile and Manhattan plots of S. aureus phagosome maturation GWAS results.

Figure 2-7: Quantile-Quantile and Manhattan plots of *S. aureus* phagosome maturation GWAS results.

QQ plots show the differences between the observed and expected -log10 (*p*-value) of the study results. The red line indicates the expected distributions under the null hypothesis. The gray shaded region represents the 95% confidence interval. (B) Manhattan plot of single nucleotide polymorphisms for the study data. X-axis represents chromosomes. Y-axis represents -log10 (p-values) obtained by genome-wide association analysis. The red line indicates the suggested threshold for genome-wide significance ($p < 1 \times 10\text{-}5$). Q-Q plots map the observed negative-log10 of each SNP *p*-value ($-\log_{10}(p)$) against the expected $-\log_{10}(p)$. Male (A), Female (C), Pooled (E) and Antagonistic effects (G) Q-Q plots are shown. Manhattan plots depict the GWAS results on a genomic scale: SNP *p*-values are plotted by genomic position and chromosome arm on the x-axis and the y-axis is the $-\log_{10}(p)$ of the p-value. Male (B), Female (D), Pooled (F) and Mixed effects (H) Manhattan plots are shown. SNPs with *p*-values $< 10^{-5}$ are plotted above the blue horizontal line at $-\log_{10}(p) = 5$. Three highly significant SNPs with *p*-values < 5.0e-08 are plotted above the horizontal red line showing $-\log_{10}(p) = 7.3$.

In an effort to detect signaling pathways that play a role in phagocytosis, we lowered our significance threshold to include SNPs with *p*-values less than $1x10^{-4}$ for each single phenotype GWA analysis. We identified an additional 201 SNPs in or near 124 genes associated with pooled sex median phagosome maturation; 186 SNPs in or near 150 genes for males and 185 additional SNPs in or near 85 genes associated with female phagosome maturation (Online Supplemental Table 2 – Table 4).

To identify candidate polymorphisms with sexually dimorphic effects in the DGRP, we carried out an additional GWA to identify SNPs that were significantly associated with the phenotypic difference between the sexes, calculated as: $female_{phagosome\ maturation} - male_{phagosome\ maturation}$. We termed such SNPs as antagonistic and hypothesized that the polymorphisms would exert different effects on males and females during the cellular immune response to *S. aureus.* We identified 47 SNPs with *p*-value $<10^{-5}$ and 30 associated genes (Online Supplemental Table 5). We also relaxed the significance threshold for mixed effects antagonistic SNPs to include all SNPs with *p*-values $< 10^{-4}$. We found an additional 258 SNPs in or near 163 candidate genes that exerted opposite effects in males and females (Online Supplemental Table 6). Overall, we obtained a list of 528 candidate genes, 18 of which are non-protein coding genes (Online Supplemental Table 7). Based on our false discovery rate estimates, about 90 genes are true associations.

C. Candidate genes implicated in immunity

Of the 528 candidate genes, six have been shown to clearly play a role in the immune response in *Drosophila.* One known phagocytic receptor, Scab (scb) was identified in our screen. *scb* encodes an integrin alpha chain (αPS_3) that partners with

Integrin βv to form a heterodimer that serves as a receptor for apoptotic cells and *S. aureus* (Nonaka et al., 2013). *Rac2,* a Rho-GTPase, was also identified and has been proposed to act downstream of *croquemort* to mediate phagocytosis of *S. aureus* (Stuart et al., 2005). *Peridoxin* 5 (*Prx5*) and *Calcineurin A at 14F* (*CanA-14F*), two genes that modulate immune-regulated signaling cascades in response to septic injury, were also identified in our screen. *Prx5* inhibits c-Jun N-terminal kinase (JNK) signaling after Gram-positive and Gram-negative bacterial infections (Radyuk et al., 2010a). *CanA-14F* regulates Toll signaling by inducing nuclear localization of Dorsal in response to Gram-positive bacterial infection (Li and Dijkers, 2015; Radyuk et al., 2010a). Two key regulators of the Toll signaling cascade, *Dorsal-related immunity factor (Dif)* and *spatzle* (*spz*), were also found in our gene list. This result was surprising in light of experimental evidence that indicates the humoral and cellular responses do not genetically interact during the Drosophila response to *S. aureus (Nehme et al., 2011).*

Nineteen of the candidate genes (*CG11208, karst, regucalcin, mbt, Cht2, cindr, spg, CG31249, Pax, Act5C, Hsp23, CaBP1, Exo70, LKR, scb, Fim, Sod2, 26-29-p,* and *nrv1*) were previously identified in a proteomic analysis of the *Drosophila* phagosome, providing further evidence of their potential roles in phagocytosis and phagosome maturation in hemocytes (Stuart et al., 2007). Of the 528 genes associated with one or more GWAS trait, 27 have human orthologs that have been associated with the immune response in humans (Table 2-3).

Table 2-3: Notable candidate genes with human orthologs implicated in immunity.

Human Trait	***D. mel.* gene**	**Human gene**	**% Identity**	***S. aureus* phagosome maturation trait and smallest associated *p*-value**	***Drosophila* experimental evidence**	**Human experimental evidence**	**Reference**
Immune response	Dif	RELA	29%	-Pooled (8.25e-05)	Toll pathway effector. Hemocyte differentiation.	Innate immune response. Hematopoesis	(Ip et al., 1993) (Huang et al., 2005) (Brown et al., 2009) (Hayden and Ghosh, 2011) (Anrather et al., 2006)
	Sod2	SOD2	62%	-Pooled (4.48e-06)	Mitochondrial superoxide dismutase. Mediates hematopoiesis. S2 cell phagosome proteome.	Respiratory burst in neutrophils.	(Sinenko et al., 2011) (Sinenko et al., 2010) (Lim et al., 2014) (Stuart et al., 2007) (Olsson et al., 2011)
	Prx5	PRDX5	57%	-Pooled (5.27e-05)	Antioxidant enzyme. Negatively regulates the JNK signaling pathway.	Implicated in Inflammatory diseases: Crohns, Alopecia areata, Sarcoidosis	(Radyuk et al., 2010a) (Radyuk et al., 2010b) (Franke et al., 2010) (Petukhova et al., 2010) (Fischer et al., 2012)

Immune response	CG42339	SBSPON	22%	-Pooled (3.91e-05)	Thrombospondin, type 1 repeat (IPR000884) & Somatomedin B domain (IPR001212)	IEA – polysaccharide binding, immune response, scavenger receptor activity, receptor-mediated endocytosis	(Kenny et al., 2012)
	CanA-14F	PPP3CA	78%	-Pooled (5.5e-06)	Phosphatase. Mediates Toll-signaling by inducing nuclear localization of Dorsal in response to Gram+	Innate immune response. NFAT dephosphory-lation.	(Dijkers and O'Farrell, 2007) (Li and Dijkers, 2015) (Bengoechea-Alonso et al., 2003)
	scb	ITGA4	25%	Antagonistic (2.35e-05)	Phagocytosis, Immune response to *S. aureus*. Heart development. S2 cell phagosome proteome.	Integrin, alpha 4 (antigen CD49D, alpha 4 subunit of VLA-4 receptor).	(Stroschein-Stevenson et al., 2006) (Nonaka et al., 2013) (Stuart et al., 2007) (Vanderploeg et al., 2012)
	Fim	LCP1	54%	-Females (9.93e-05)	Actin binding. Calcium binding. S2 cell phagosome proteome.	Lymphocyte cytosolic protein 1 (L-plastin). Actin crosslinking at immune phagocytic cup.	(Stuart et al., 2007) (Janji et al., 2006)
	Rac2	RAC1 RAC2	82% 88%	-Males (3.42e-05)	Phagocytosis of *S. aureus, E.coli,* & *Pseudomonas aeruginosa*. S2 cell phagosome proteome.	Regulates Fc receptor mediated phagocytosis in macrophages and neutrophils. Neutrophil immune-deficiency syndrome.	(Stuart et al., 2005) (Stuart et al., 2007) (Avet-Rochex et al., 2007) (Sampson et al., 2012) (Castellano et al., 2000) (Forsberg et al., 2003)

	Lectin-galC1	CLEC4D	28%	-Males (8.57e-05)	*E. coli* agglutination.	Monocyte/ Macrophage endocytic receptor.	(Tanji et al., 2006) (Arce et al., 2004)
	CG6145	NADK	51%	-Males (5.06e-06)	Ortholog of human NAD kinase.	NAD kinase. Key enzyme regulating cellular NADPH levels	(Pollak et al., 2007)
Cytoskeletal organization	sif	TIAM1	31%	-Pooled (1.82e-05) -Females (2.91e-05)	Rac/Rho-Guanyl-nucleotide exchange factor activity. Mediates Rac/SCAR/Arp2/3 actin nucleation.	Macrophage and Dendritic Cell actin dynamics.	(Sone et al., 1997) (Georgiou and Baum, 2010) (Bohdanowicz et al., 2013)
	CG32082	BAIAP	28%	Antagonistic (3.76e-05) - Pooled (3.82e-05) -Males (6.81e-05)	BAR-domain protein (IPR027681)	Actin cytoskeletal dynamics and formation of membrane protrusions.	(Scita et al., 2008)
	Pkc98E	PRKCE	58%	-Pooled (5.92e-06)	Protein kinase C family member.	Recruited to phagosome by DAG. IgG-dependent phagocytosis. Important for LPS-mediated signaling in activated macrophages.	(Castrillo et al., 2001) (Cheeseman et al., 2006)
	Ziz	DOCK9	39%	-Males (4.57e-05)	Rho-GEF.	Zizimin1, CDC42 activator.	(Meller et al., 2002) (Meller et al., 2005)
	mbt	PAK4 PAK7	50% 47%	-Pooled (7.06e-05)	Regulates actin cytoskeletal dynamics. PAK family of Ser/Thr protein kinase.	Regulates actin cytoskeletal dynamics. Effector of Rac and CDC42.	(Schneeberger, 2003) (Wells and Jones, 2010) (Stuart et al., 2007)

Vesicle trafficking	CG15087	VPS51/ ANG2	42%	-Males (9.71e-05)		Vesicle-recycling. Member of Golgi-associated retrograde protein (GARP) complex.	(Pérez-Victoria et al., 2010)
	rg	LRBA	40%	-Pooled (7.93e-05)	Protein kinase A anchor protein. Interacts with Notch and EGFR signaling pathways.	Vesicle trafficking in response to LPS stimulation in murine and human macrophages.	(Shamloula et al., 2002) (Wang et al., 2001) (Alangari et al., 2012)
	kst	SPTBN5	32%	-Pooled (7.34e-07) -Males (8.01e-05)	Endocytosis and endosomal transport. S2 cell phagosome proteome		(Phillips and Thomas, 2006) (Tjota et al., 2011) (Stuart et al., 2007)
	Exo70	EXOC7	31%	Antagonistic (1.48e-05)	Exocyst complex. Phagocytosis of bacteria. S2 cell phagosome proteome.	Vesicle trafficking. Interacts with Cdc42 to regulate phagosome formation during phagocytosis	(Stuart and Ezekowitz, 2005) (Stuart et al., 2007) (Mohammadi and Isberg, 2013) (Guichard et al., 2014)
	Syt12	SYT12	32%	-Males (8.3e-07) -Pooled (3.32e-06) -Females (1.02e-05)	Synaptic vesicle release.	Membrane trafficking.	(Adolfsen and Littleton, 2001) (Adolfsen et al., 2004) (Maximov et al., 2007)

Lysosomes	CG8596	MFSD8	35%	-Pooled (7.73e-05) -Males (8.85e-05)	Major Facilitator Superfamily Protein.	Localizes to lysosomal membrane. Lysosomal storage disorder.	(Mole et al., 2005)
	Lip4	LIPA	38%	- Females (4.78e-05) Antagonistic (2.28e-05)	Acid Lipase. Triglyceride lipase activity.	Lipase A, lysosomal acid, cholesterol esterase. Lysosomal enzyme.	(Vihervaara and Puig, 2008) (Anderson and Sando, 1991) (Garin et al., 2001)
	CG14291	SGSH	55%	Antagonistic (9.82e-07)	N-sulfoglucosamine sulfohydrolase activity.	Enzyme involved in lysosomal degradation of heparan	(Scott et al., 1991)
	LManIII/ CG9463	MAN2B1	39%	-Males (8.66e-05)	Alpha-mannosidase activity, carbohydrate binding.	Mannosidase enzyme involved in lysosomal glycoprotein turnover.	(Rosenbaum et al., 2014)
	ATP7	ATP7B ATP7A	47% 46%	-Males (6.83e-07) -Pooled (1.18e-05)	Copper efflux/transport in S2 cells.	Wilson's Disease. Co-localizes with Rab7 and late endosomes. Transport copper from cytosol to late endosomal lumen.	(Southon et al., 2004) (Harada et al., 2005)
	Cht2	CHIT1	35%	-Pooled (4.52e-05)	Carbohydrate metabolic process, chitinase activity.	Lysosomal storage disease.	(Stuart et al., 2007) (Boot et al., 1998)

Table 2-3: Notable candidate genes with human orthologs implicated in immunity.

Candidate genes associated with immunity, cytoskeletal organization, vesicle trafficking and lysosome dynamics in *Drosophila* or humans.

D. Gene Ontology Analysis

To evaluate whether genes implicated in *S. aureus* phagosome maturation are functionally related, we completed gene ontology (GO) enrichment analysis using FlyMine v40.1 (Lyne et al., 2007). All GO analyses were carried out using the Holm-Bonferroni multiple testing correction, and GO terms with corrected *p*-values < 0.05 are reported. No GO categories were overrepresented for the 185 candidate genes (SNP *p*-values < $1x10^{-4}$) associated with phagosome maturation in males, but several GO categories were found for female, pooled and antagonistic associated genes. GO analysis of the 103 female phagosome maturation associated genes (SNP *p*-values < $1x10^{-4}$) showed overrepresentation of two biological process GO terms: cell fate commitment (*p*-value 0.019) and anatomical structure morphogenesis (*p*-value 0.022). The female associated gene list was also enriched for two cellular component GO terms: cell periphery (*p*-value 0.0126) and plasma membrane associated proteins (*p*-value 0.024). The list of 145 candidate genes identified in the pooled GWA was enriched for 21 biological process GO terms, including biological regulation (*p*-value 1.56e-05), signaling (*p*-value 0.0017), cell projection organization (*p*-value 0.0046), and cell surface receptor signaling pathway (*p*-value 0.023). One additional molecular function category was enriched in pooled candidate genes: sequence-specific DNA binding (*p*-value 0.023). Like the female GO analysis, the cellular component GO terms for pooled candidate genes, included plasma membrane (*p*-value 3.22e-06) and cell periphery (*p*-value 6.64e-05). Biological process GO enrichment analysis for 194 candidate genes (SNP *p*-values < 10^{-4}) associated with antagonistic phagosome maturation effects revealed overrepresentation of two GO terms: locomotion (*p*-value 0.02) and movement of cell or

subcellular component (*p*-value 0.045). Proteins with IgG-like folds were also found to be overrepresented in the antagonistic GWA candidate gene list (*p*-value 0.001).

We also carried out GO analysis using our complete list of 528 candidate genes and uncovered enrichment of 82 biological process GO terms (Online Supplemental 8). Among the most significantly enriched categories were generation of neurons (*p*-value 3.99e -10), cell projection organization (*p*-value 1.00e -06), and locomotion (*p*-value 5.29e -06). These results suggest that a large subset of candidate genes associated with *S. aureus* phagosome maturation may play dual roles in regulating hemocyte and neuronal cytoskeletal dynamics. Additional terms of interest included potassium ion transport (*p*-value 0.01) and signaling (*p*-value 0.05). Furthermore, eight candidate genes have been previously characterized as important mediators related to lymph gland development (*p*-value 0.04): *zfh1, lz, Pvf3, Ser, Dif, tin, Rac2 and dpp*. This finding indicates that the functional read-out of the screen, pHrodo-*S. aureus* intensity of the hemocytes along the dorsal vessel, also results in the identification of SNPs affecting hemocyte development and dorsal vessel morphology. The molecular process enrichment analysis identified eleven candidate genes important for potassium ion transmembrane transporter activity (*p*-value 0.003): *slo, SK, CG42732, Nckx30C, Sh, Shab, Ork1, Elk, Task6, Nha2,* and *nrv1,* suggesting that phagocytic membrane potential and ion flow may be important factors during phagosome maturation in *Drosophila* hemocytes. Finally, cellular component GO terms for plasma membrane associated proteins were overrepresented (smallest *p*-value 1.92e -09) and protein domain enrichment analysis revealed that our candidates were enriched for immunoglobulin-like (IgG-like) proteins (*p*-value 2.9e-05).

E. Candidate genes associated with Oxidative Stress Resistance

Fifty-four genes in the *S. aureus* phagosome maturation candidate list were also identified as associated with oxidative stress resistance in another DGRP study (Weber et al., 2012) (*p*-value 4.28e-09). The oxidative stress resistance GWAS was enriched for processes associated with neuronal development, genes associated with ion transport, and genes encoding proteins with IgG-like domains. The high degree of similarity between the two studies may indicate that many of the factors that govern resistance to oxidative stress at the level of the whole organism also mediate the oxidative burst in hemocytes. Reactive oxygen species (ROS) are integral to bacterial killing within the phagolysosome. Conversely, overproduction of ROS within phagocytic cells causes immune dysfunction and flies with defective antioxidant production show impaired cellular immune responses. For example, hemocytes of flies with a disrupted *polyph* gene (a glutamate transporter) exhibit overproduction of ROS in response to *S. aureus* and *polyph* mutant blood cells show a decreased ability to phagocytose *S. aureus* (Gonzalez et al., 2013). Thus, it is possible that some of the fifty-four genes identified in our screen and the oxidative stress GWA may be participate in the oxidative burst process during phagocytosis in hemocytes.

F. Functional tests to validate candidate genes

To confirm the effects of a subset of our candidate genes on *S. aureus* phagosome maturation, we repeated the *in vivo S. aureus* phagosome maturation assay using adults expressing RNAi knockdown constructs to silence genes specifically in hemocytes. We chose to validate 38 of the 528 genes related to SNPs with p-values less than $1x10^{-4}$ based

on the availability of TRiP (http://www.flyrnai.org) and VDRC (http://www.stockcenter.vdrc.at) RNAi stocks. We tested the effects of three candidate genes whose corresponding SNPs had high statistical significance (p-value < 5.0e-08): *bif, bbc,* and *CG42673.* In addition, we selected 15 genes that encoded proteins predicted to have IgG-like domains, 12 genes that encode proteins predicted to be at the plasma membrane, and 8 proteins previously found in the proteomic analysis of the *Drosophila* phagosome (Stuart et al., 2007).

The results for the secondary RNAi screen are presented in Table 2-4. For 28 candidate genes, mRNA knockdown in hemocytes did not affect phagocytosis of *S. aureus*. We speculate that due to the high false discovery rate of our GWA, many of the genes we tested are false positive hits. Genes that truly play a role in phagosome maturation in the DGRP may not validate using RNAi due to the fact that hemocyte-specific RNAi may not recapitulate the effect of the associated SNPs in the DGRP. Within the natural population, SNPs may not confer loss of function phenotypes or SNPs may alter function of associated genes in other tissues. Ten candidate genes did influence *S. aureus* phagosome maturation during the initial tests. Two follow-up *in vivo S. aureus* phagosome maturation assays were then performed. Ultimately, three of the candidate genes tested with RNAi showed significantly decreased *S. aureus* phagosome maturation phenotypes: *defective in proboscis extension response 10 (dpr10)*, *friend of echinoid* (*fred*), and *CG42673*. Our studies were then expanded to ascertain the extent of immune-related dysfunction conferred by loss of each of the 3 genes.

Table 2-4: Candidate genes tested in secondary screen using RNA interference in hemocytes.

Category	Gene Symbol	GWAS parameter and smallest *p*-value	Protein features	Hemocyte-specific RNAi phenotype	Follow-up (n= 3 experiments) & results
Top *p*-value	***bif***	**Antagonistic effects**: 9.4e-09	-Cytoskeleton binding protein	No phenotype.	
	bbc	**Pooled**: 2.44e-08 **Males**: 5.69e-06	-CDP-alcohol phosphatidyl-transferase	No phenotype.	
	CG42673	**Female:** 9.66e-08 **Male:** 9.07e-05	-Pleckstrin homology-like domain	M & F decreased phagosome maturation	M and F decreased phagosome
IgG-like domain	***fas***	**Pooled**: 4.5e-05 **Males**: 9.44e-05 **Females**: 1.45e-05 **Antagonistic effects**:3.4e-08	-IgG-like domain	M & F Reduced phagosome maturation	No phenotype.
	dpr1	**Pooled**: 3.95e-05 **Females**: 8.87e-06 **Antagonistic effects**:1.96e-06	-IgG-like domain	No phenotype.	
	dpr8	**Male**: 4.87e-05 **Antagonistic effects**: 5.6e-07	-IgG-like domain	No phenotype.	
	DIP-α CG32791	**Male**: 7.642e-06	-IgG-like domain -DPR interacting protein	No phenotype.	
	CG5984	**Male**: 2.7e-06	-IgG-like domain -Actin binding Filamin-B	No phenotype.	
	CG12484	**Female:** 4.68e-05	-IgG-like domain	No phenotype.	
	CG34113	**Female:** 1.01e-05	-IgG-like domain	No phenotype.	
	fred	**Female:** 7.48e-05	-IgG-like domain	F decreased phagosome maturation	F decreased phagosome maturation

	side	**Male**: 5.67e-05 **Female:** 3.77e-05	-IgG-like domain	No phenotype.	
	CG31814	**Antagonistic effects:** 4.96e-05	-IgG-like domain	No phenotype.	
	dpr10	**Antagonistic effects:** 2.3e-05	-IgG-like domain	F decreased phagosome maturation	F decreased phagosome maturation
	robo3	**Antagonistic effects**: 6.15e-05	-IgG-like domain	No phenotype.	
	kirre	**Male**: 5.65e-05 **Antagonistic effects**: 2.28e-05	-IgG-like domain	No phenotype.	
	beat-VII	**Antagonistic effects**: 7.12e-05	-IgG-like domain	F Increased phagosome maturation	No phenotype.
	CG34353	**Antagonistic effects**: 8.36e-06	-IgG-like domain	No phenotype.	
	ATP7	**Pooled**: 1.18e-05 **Males**: 6.82e-07	-Copper transport	No phenotype.	
Predicted to localize to plasma membrane	***FMRFaR***	**Female**: 1.8e-05 **Antagonistic effects**: 3.65e-05	-G protein coupled receptor, rhodopsin-like	No phenotype.	
	loco	**Female**: 2.48e-07 **Antagonistic effects**: 1.97e-05	-G-protein alpha-subunit binding	No phenotype.	
	Sh	**Female**: 2.98e-06 **Antagonistic effects**: 8.93e-06	-Potassium channel	No phenotype.	
	GluRIB	**Pooled**: 3.95e-05 **Males**: 4.09e-05 **Antagonistic effects**: 7.44e-06	-Glutamate receptor	No phenotype.	
	CG31760	**Antagonistic effects**: 7.62e-05	-GPCR	No phenotype.	
	Ork1	**Antagonistic effects**: 4.09e-05	-Potassium Channel activity	No phenotype.	
	Con	**Antagonistic effects**: 1.04e-05	-Leucine-rich repeats	No phenotype.	
	foi	**Antagonistic effects**: 3.28e-05	-Zinc/iron permease	No phenotype.	
	Toll-6	**Antagonistic effects**: 4.77e-05	-TIR domain	M Reduced phagosome	No phenotype
	rk	**Antagonistic effects**: 2.97e-05	-GPCR receptor activity	M & F Reduced phagosome maturation	No phenotype.
	Sema-1b	**Antagonistic effects**: 1.66e-05	-Semaphorin	M & F Reduced phagosome maturation	No phenotype.
	kst	**Pooled**: 7.34e-07 **Males**: 8.01e-05	-Cytoskeleton binding protein	No phenotype.	

S2 cell phagosome proteome	***nrv1***	**Pooled**: 5.6e-05 **Males**: 7.6e-06	-Na+/K+ ATPase subunit	No phenotype.	
	regucalcin	**Pooled**: 5.47e-05 **Males**: 3.95e-05	-Intracellular signaling	No phenotype.	
	LKR	**Male**: 6.9e-05 **Antagonistic effects**: 5.77e-05		M Reduced phagosome maturation	No phenotype.
	Pax	**Male:** 8.57e-05		No phenotype.	
	Fim	**Female**: 9.3e-05	-Actin binding	No phenotype.	
	Exo70	**Antagonistic effects**: 1.48e-05	-Exocyst complex protein	No phenotype.	
	Hsp23	**Antagonistic effects**: 9.77e-05	-Heat shock protein	No phenotype.	

Table 2-4: Candidate genes tested in secondary screen using RNA interference to silence expression in hemocytes.

Genes were selected if they met one of the following criteria: Top *p*-value for a given GWA parameter, encode a protein with IgG-like domain, encode for a protein predicted to localize to the plasma membrane, protein found in the Stuart *et al.* 2007 S2 cell phagosome proteome. Male and female adult flies with the candidate genes silenced in hemocytes were tested for *S. aureus* phagosome maturation.

G. Dpr-family member *dpr10* affects S. aureus phagosome maturation

Dpr1 is the founding member of a family of 20 paralogous IgG-like proteins in *Drosophila* (Nakamura et al., 2002). Nakamura *et al.* characterized a role for *dpr1* in the behavioral response to salty foods. The absence of *dpr1* in a subset of neurons in the gustatory organs causes *dpr1* mutant flies to exhibit defects in the salt aversion, indicating a role for the extracellular IgG-like domains of dpr in the perception of small chemical stimuli. Our study found 4 members of the Dpr-family, *dpr1*, *dpr6*, *dpr8*, and *dpr10*, are associated with *S. aureus* phagosome maturation (Table 2-5). Of theses genes, *dpr6* and *dpr10* are the most closely related. Dpr6 and Dpr10 share 67% identity at the amino acid level. Furthermore, *dpr6* and *dpr10* are clustered on the 3rd chromosome, but are transcribed in opposite orientations. The SNPs associated with each of these genes are located within introns and it is unclear how the expression of the genes is affected by the presence of the minor allele variants. Recently, a study characterizing the extracellular domains of cell surface receptors and ligands showed direct interactions between the Dpr-family members and several previously novel IgG-like domain-containing proteins (Ozkan et al., 2013). An additional gene identified in our screen, *CG32791*, also known as *Dpr-interacting protein α* (*DIP*-α), was shown to physically interact with *dpr6* and *dpr10* (Ozkan et al., 2013). Owing to the availability of RNAi lines, we were able to assess the effects of *dpr1, dpr8, dpr10* and *DIP-α* but not *dpr6.* Of these genes, only *dpr10* hemocyte-specific knockdown led to decreased pHrodo-*S. aureus* fluorescence in adult hemocytes (Figure 2-8).

Dpr family gene and genomic location	GWAS parameter	SNP ID	*P*-value	Minor Allele	Major Allele	Minor Allele Frequency
dpr1 2R:16,585,988…16,637,947 [+]	Pooled	2R_16694360	3.95E-05	C	T	0.06098
	Sex difference	2R_16638854	1.96e-06	T	A	0.2892
	Females	2R_16647300	1.61E-05	A	G	0.2716
		2R_16647718	8.87E-06	G	A	0.2099
dpr6 3L:9,967,751…10,132,460 [+]	Pooled	3L_9992040	2.18E-05	A	G	0.1519
		3L_9992510	2.25E-05	G	A	0.2143
dpr8 X:14,216,259…14356,384 [+]	Males	X_14204940	4.87E-05	C	A	0.3676
		X_14206158	8.30E-05	G	A	0.4416
		X_14207330	6.13E-05	C	A	0.4878
	Sex difference	X_14225948	1.45e-05	G	A	0.3429
		X_14225998	5.6e-07	A	T	0.3065
		X_14226122	3.9e-05	C	A	0.338
		X_14226134	6.9e-05	C	T	0.4359
dpr10 3L:10,140,554…10,179,208 [-]	Sex difference	3L_10165573	2.4e-05	A	T	0.3171

Table 2-5: List of Dpr-family genes associated with S. aureus phagosome maturation in the DGRP.

There are 20 paralogous Dpr-family genes in *Drosophila melanogaster.* Four members of the family were identified in the *S. aureus* phagosome maturation GWAS. Dpr-family genes exhibit sexually dimorphic effects on the cellular immune response.

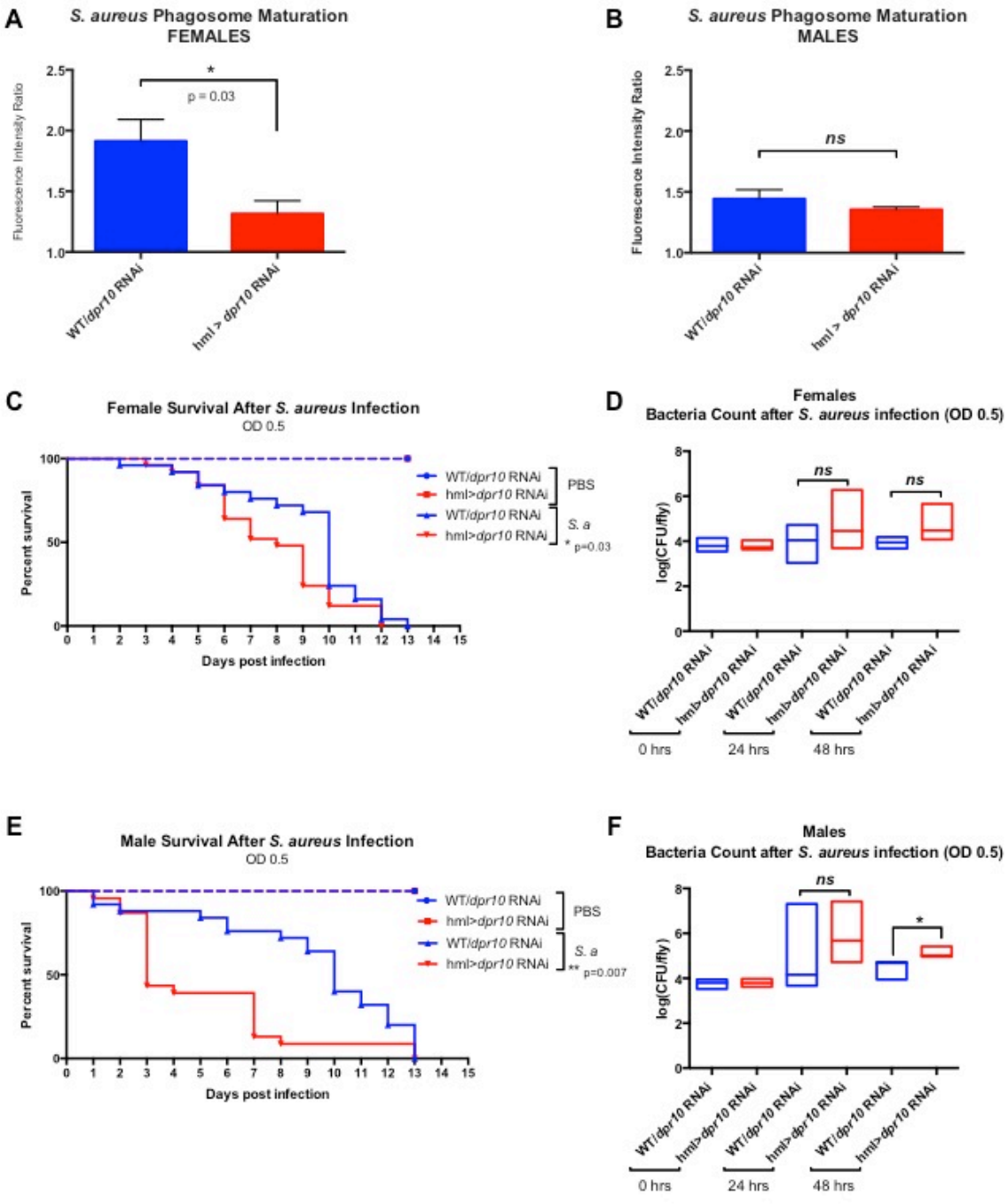

Figure 2-8: *dpr10* RNAi affects S. aureus phagosome maturation in adult hemocytes and leads to decreased resistance to *S. aureus* septic infection.

Figure 2-8: *dpr10* RNAi affects *S. aureus* phagosome maturation in adult hemocytes and leads to decreased resistance to *S. aureus* septic infection.

(A) Quantification of the phagosome maturation of pHrodo-labeled *S. aureus* bioparticles in female WT/*dpr10* RNAi and hml>*dpr10* RNAi flies. (B) Quantification of the phagosome maturation of pHrodo-labeled *S. aureus* bioparticles in male WT/*dpr10* RNAi and hml>*dpr10* RNAi flies. Six to eight flies per genotype were tested in each experiment. Experiments were performed at least 3 times. (C) Representative survival curve of female WT/*dpr10* RNAi and hml>*dpr10* RNAi flies after injection of *S. aureus* (OD 0.5). n=24-30 flies. Experiments were performed in triplicate. (D) *S. aureus* (OD 0.5) bacterial load in female WT/*dpr10* RNAi and hml>*dpr10* RNAi flies at 0, 24, and 48 hours post-infection. Bacterial load was measured in 4-6 individual flies per genotype at each time point and the experiment was performed in triplicate. (E) Representative survival curve of male WT/*dpr10* RNAi and hml>*dpr10* RNAi flies after injection of *S. aureus* (OD 0.5). n=24-30 flies. Experiments were performed in triplicate. (F) *S. aureus* (OD 0.5) bacteria load in female WT/*dpr10* RNAi and hml>*dpr10* RNAi flies at 0, 24, and 48 hours post-infection. Bacterial load was measured in 4-6 individual flies per genotype at each time point and the experiment was performed in triplicate. Error bars, ±SE. * *p*-value <0.05, *ns* = not significantly different.

One *dpr10* associated SNP (*p-value* of 2.43e-05) was identified to have antagonistic effects on *S. aureus* phagosome maturation and was located at genomic position 3L_10165573, in the longest intron of all 4 *dpr10* isoforms (based on Flybase release 5.57). Knockdown of *dpr10* in hemocytes inhibited *S. aureus* phagosome maturation specifically in females (Figure 2-8A and B) but led to increased susceptibility to *S. aureus* septic injury in both female and male flies (Figure 2-8C and E). At 24 hours post infection, we did not see differences in *S. aureus* bacteria load in females or males. However, we did observe increased bacteria loads at 48 hours post infection in males, but not in females – although female flies did exhibit a trend toward higher bacteria loads at 48 hours. These results indicate *dpr10* modulates the immune response to *S. aureus* in a sex-specific manner, causing reduced tolerance infection in females but leading to decreased resistance in males (Figure 2-8D and F).

To differentiate between pathogen recognition and downstream phagosome maturation processes, we also performed an *in vivo S. aureus* phagocytosis assay. We observed no differences between hml>*dpr10* RNAi and WT/*dpr10* RNAi males or female flies (Figure 2-9). Additionally, we saw no defects in phagocytosis of 1μm latex beads or phagosome maturation of pHrodo-conjugated *E. coli* bioparticles (Figure 2-9). Together, these results establish a functional role for *dpr10* in regulating the phagosome maturation of *S. aureus* in Drosophila.

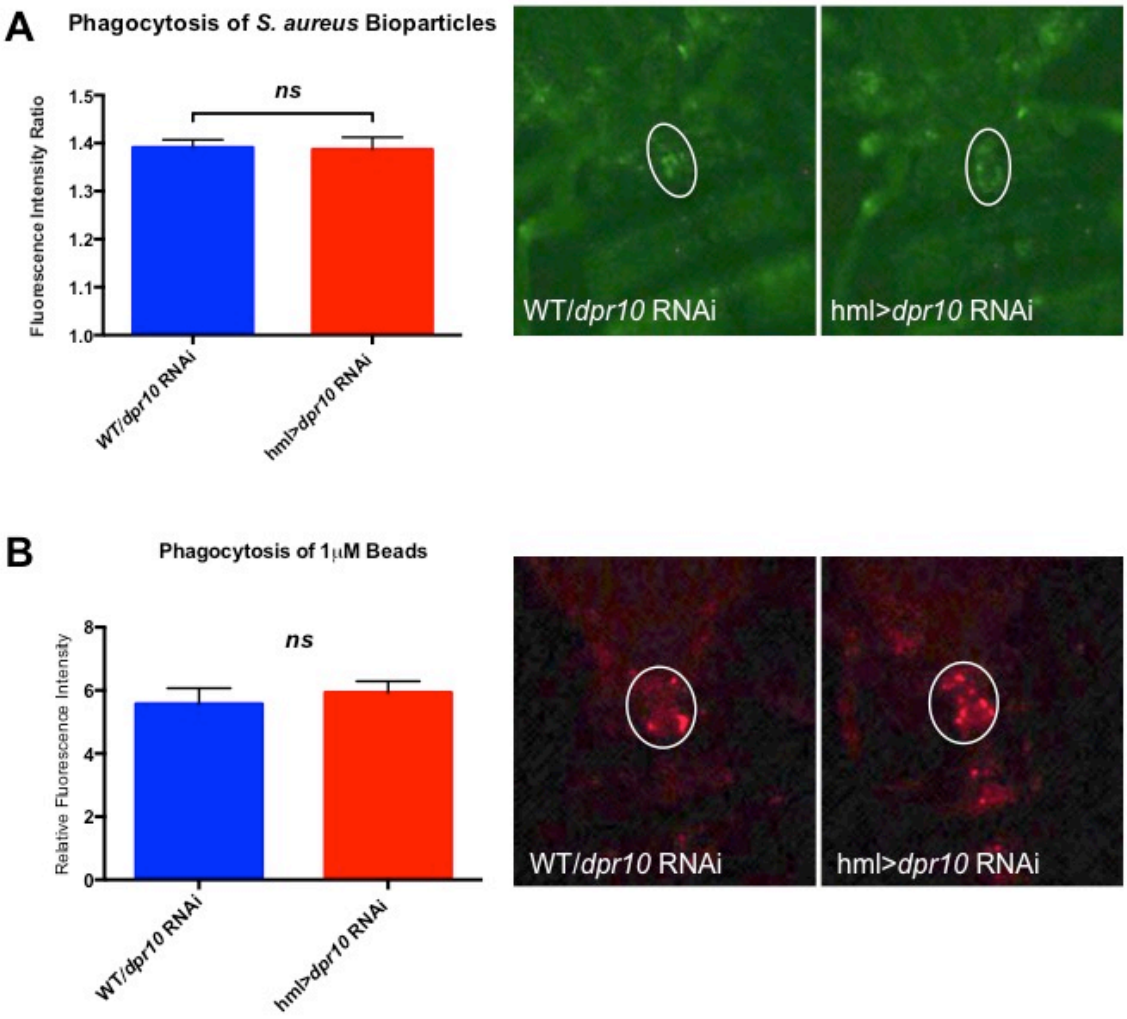

Figure 2-9: *dpr10* RNAi does not affect phagocytosis of *E. coli* or latex beads.

(A) Quantification of the phagocytosis of *S. aureus* bioparticles in WT/*dpr10* RNAi and hml>*dpr10* RNAi flies. (B) Quantification of the phagocytosis of 1μM latex beads in WT/*dpr10* RNAi and hml>*dpr10* RNAi flies. Six to eight flies per genotype were tested in each experiment. Experiments were performed at least 3 times. Error bars, ±SE. * *p*-value <0.05, *ns* = not significantly different.

H. *Friend of echinoid* RNAi leads to decreased *S. aureus* phagosome maturation

The cell adhesion molecule (CAM), *friend of echinoid* (*fred*), was found to be associated with *S. aureus* phagosome maturation in the female GWAS. *fred* encodes a plasma membrane-associated protein with seven immunoglobulin-C2 type domains, two Fibronectin type-III domains, a transmembrane domain and an intracellular region (Chandra et al., 2003). *fred* and its paralog, *echinoid* (*ed*), are located on chromosome arm 2L, proximal to one another, but in opposite orientations (Chandra et al., 2003). *ed* and *fred* genetically interact with the Notch signaling pathway as well as the epidermal growth factor (Egfr) signaling pathway (Chandra et al., 2003; Fetting et al., 2009). *fred* was shown to coordinate cellular movement during ommatidial rotation and during wing disc sensory organ development.

Silencing *fred* expression in hemocytes led to reduced *S. aureus* phagosome maturation in female flies (Figure 2-10A and B). As was observed with *dpr10* RNAi flies, both female and male hml > *fred* RNAi flies were more susceptible to *S. aureus* infections than control flies (Figure 1-10C and E). However, in contrast to the tolerance phenotype observed in *dpr10* RNAi flies, *fred* RNAi led to decreased resistance to *S. aureus* infection (as both male and female flies carried higher bacteria loads at 24 hours post infection) (Figure 2-10D and F). To determine the extent of cellular immune defects caused by *fred* RNAi loss-of-function, we tested for phagocytosis of fluorescein labeled *S. aureus* bioparticles, 1μm latex beads, and fluorescein-conjugated *E. coli* bioparticles. We saw no differences in any of these assays between control and *fred* RNAi flies (Figure 2-11). Together, these results demonstrate that *fred* plays a more specific role in *S. aureus* phagosome maturation.

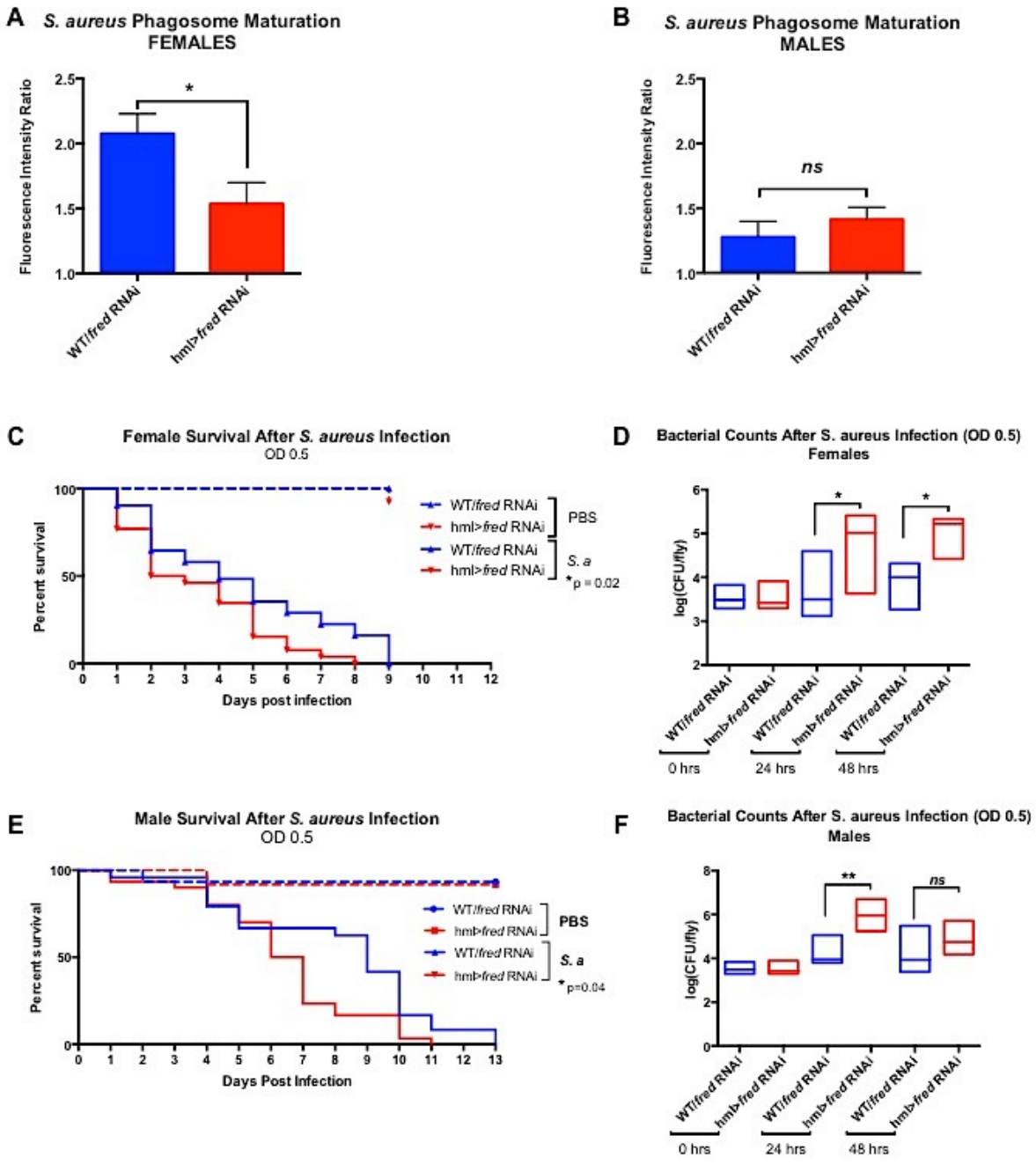

Figure 2-10: *fred* RNAi affects *S. aureus* phagosome maturation in adult hemocytes and leads to reduced resistance to *S. aureus*.

Figure 2-10: *fred* RNAi affects *S. aureus* phagosome maturation in adult hemocytes and leads to reduced resistance to *S. aureus*. (A) Phagosome maturation of pHrodo-labeled *S. aureus* bioparticles in female WT/*fred* RNAi and hml>*fred* RNAi flies. (B) Phagosome maturation of pHrodo-labeled *S. aureus* bioparticles in male WT/*fred* RNAi and hml>*fred* RNAi flies. (C) Survival of female WT/*fred* RNAi and hml>*fred* RNAi flies after injection of *S. aureus* (OD 0.5). n=24-30 flies. (D) *S. aureus* (OD 0.5) bacterial load in female WT/*fred* RNAi and hml>*fred* RNAi flies at 0, 24 and 48 hours post-infection. (E) Survival of male WT/*fred* RNAi and hml>*fred* RNAi flies after injection of *S. aureus* (OD 0.5). n=24-30 flies. (F) *S. aureus* (OD 0.5) bacterial load in male WT/*fred* RNAi and hml>*fred* RNAi flies at 0, 24, and 48 hours post-infection. All experiments were performed at least 3 times. Bacterial load was measured in 6 individual flies per genotype at each time point.. Error bars, ±SE. * *p*-value <0.05, *ns* = not significantly different.

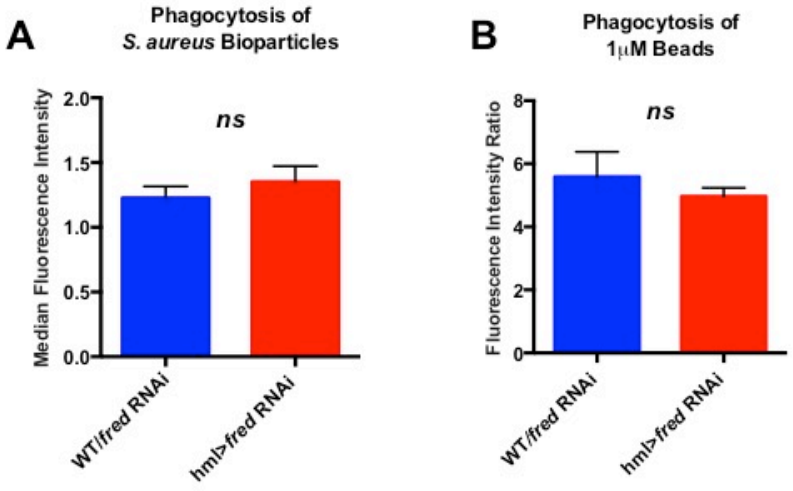

Figure 2-11: *fred* RNAi does not affect phagocytosis of E. coli or latex beads.

(A) Quantification of the phagocytosis of *S. aureus* bioparticles in WT/*fred* RNAi and hml>*fred* RNAi flies. (B) Quantification of the phagocytosis of 1μM latex beads in WT/*fred* RNAi and hml>*fred* RNAi flies. Six to eight flies per genotype were tested in each experiment. Experiments were performed at least 3 times. Error bars, ±SE. * *p*-value <0.05, *ns* = not significantly different.

I. *CG42673* RNAi in hemocytes impairs *S. aureus* phagocytosis

SNPs associated with the gene *CG42673* were identified in the female (*p*-value 9.66e-08) and male (*p*-value 9.07e-05) GWAS analyses. *CG42673* is located on chromosome arm 3L, and is one of 7 Drosophila proteins in the Disabled/Numb related adaptor family (PTHR11232). The gene is conserved in Drosophila species and three isoforms are generated via alternative splicing in *Drosophila melanogaster*. Two isoforms, *CG42673*-RB and *CG42763-RD,* contain a Capon-like N-terminal phosphotyrosine-binding domain (PTB_Capon-like: NCBI cd10270) from amino acids 1-180 – a conserved domain commonly found in signaling proteins. The PTB_Capon-like domain of CG42673 is a member of the Dab-like phosphotyrosine interaction domain family (Dab-like PID: IPR006020). Proteins with Dab-like PID domains often mediate endocytosis/processing or exocytosis and PTB domain-containing proteins function as adaptor proteins (Yaffe, 2002). These domains have a common eukaryotic pleckstrin-homology fold (PH-fold) that binds to phosphatidylinositide head groups and peptides. The third isoform, *CG42673-RC,* has a small pleckstrin-homology binding domain (PH-1 domain: NCBI cl17171) from amino acids 352-386, but this isoform lacks the full-length Dab-like PID domain found in the CG42673-PA and –PD (Figure 2-12F).

The founding member of the PTB_Capon-like domain family is the human protein CAPON, also known as Nitric oxide synthase adaptor protein-1 (NOSAP1). CAPON has a C-terminal PDZ-domain that binds to neuronal nitric oxide synthase (nNOS). The N-terminal PTB domain of CAPON binds to a small monomeric GTPase, Dexras1 (also called RASD1) (Fang et al., 2000). CG42673 lacks a C-terminal PDZ domain but has the PTB and pleckstrin homology domains common to

phosphatidylinositide binding proteins. The N-terminal PTB domains of human CAPON and CG42673-PB/-PD share 45% identity and the N-terminal domains CAPON and CG42673-PC are 33% identical over the PH-like fold (Figure 2-12F). Thus, CG42673 is a putative adaptor protein that may function as a molecular scaffold during phagocytosis of *S. aureus.*

RNAi silencing of *CG42673* in hemocytes impaired phagocytosis and phagosome maturation of *S. aureus* but did not affect fluorescein-labeled *E. coli* phagocytosis or pHrodo-*E. coli* phagosome maturation (Fig 2-12 and data not shown). The effects were similar in male and female flies, and flies of both sexes were also more susceptible to *S. aureus* infection than controls. The susceptibility to *S. aureus* infection was accompanied by an increase in bacteria load at 48 hours, suggesting that loss of *CG42673* results in decreased resistance to infection. Further work will be required to determine the importance of each specific isoform during the innate immune response to *S. aureus.*

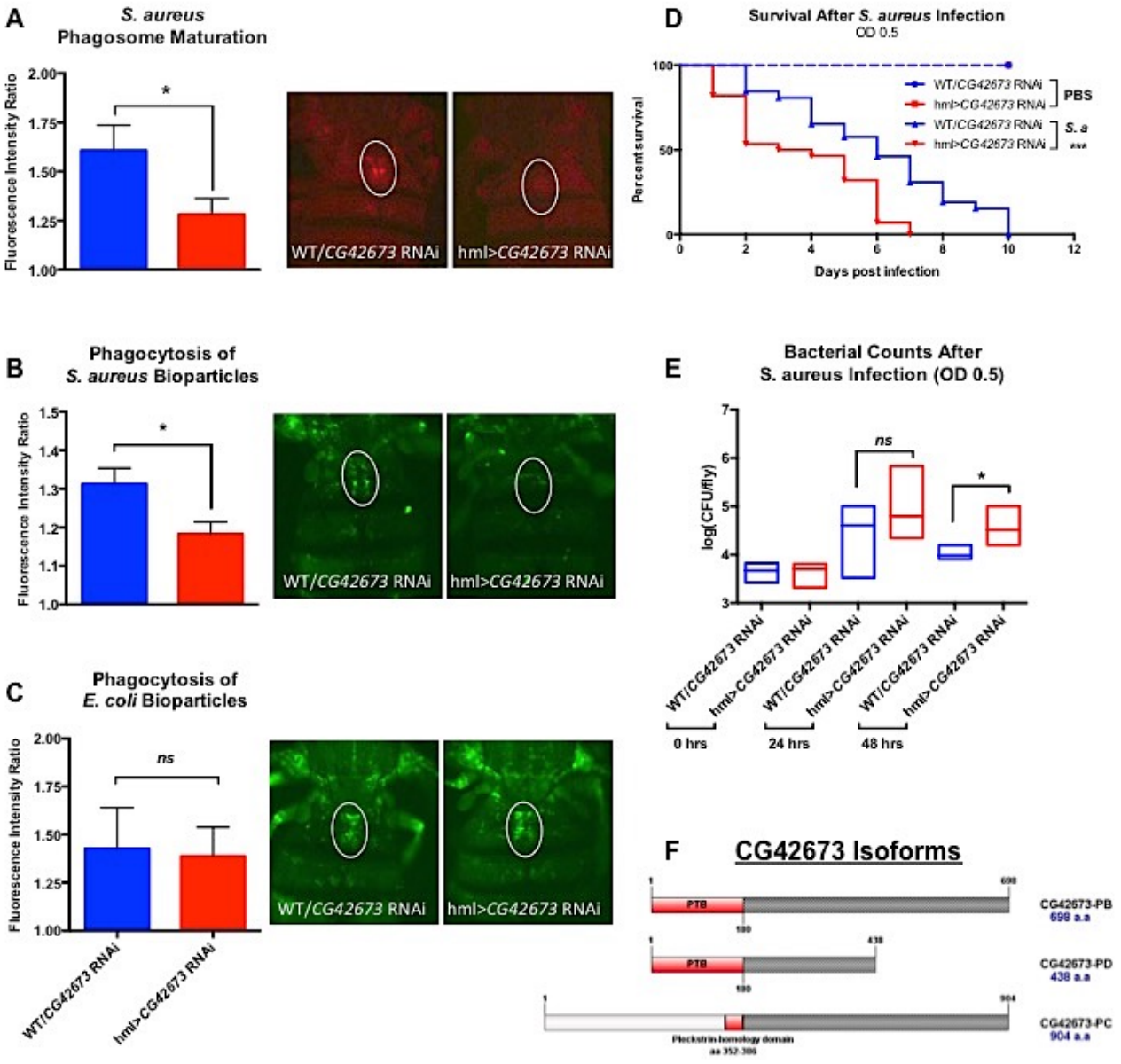

Figure 2-12: RNAi-mediated silencing of CG42673 in hemocytes causes phagocytosis defects and decreased resistance to S. aureus infection.

Figure 2-12: RNAi-mediated silencing of *CG42673* in hemocytes causes phagocytosis defects in and decreased resistance to *S. aureus* infection. (A) Phagosome maturation of pHrodo-labeled *S. aureus* bioparticles in WT/*CG42673* RNAi and hml>*CG42673* RNAi flies. (B) Phagocytosis of fluorescein-labeled *S. aureus* bioparticles in WT/*CG42673* RNAi and hml>*CG42673* RNAi flies. (C) Phagocytosis of fluorescein-labeled *E. coli* bioparticles in WT/*CG42673* RNAi and hml>*CG42673* RNAi flies. (D) Representative survival curve of WT/*CG42673* RNAi and hml>*CG42673* RNAi flies after injection of *S. aureus* (OD 0.5). n=24-30 flies. (E) *S. aureus* (OD 0.5) bacteria load in WT/*CG42673* RNAi and hml>*CG42673* RNAi flies. (F) The three isoforms of *CG42673* are translated into proteins of varying length and composition: CG42673-PB (698 amino acids), CG42673-PD (438 amino acids), and CG42673-PC (904 amino acids). Two isoforms, CG42673-PB and –PD, have a full-length N-terminal phosphotyrosine-binding domain (PTB) and CG42673-PC has a short, central pleckstrin-homology binding domain. Error bars, ±SE. * *p*-value <0.05, *ns* = not significantly different

IV. Discussion

We have taken advantage of the natural variation within the Drosophila Genetic Reference Panel to identify polymorphisms that affect *S. aureus* recognition and uptake by hemocytes. Previous mutant and RNA interference screens have found mutations in several genes that serve as phagocytic receptors. We identified a total of 528 candidate genes, many of which are novel. Given that the DGRP is a living library of polymorphisms across the entire genome, we present this *in vivo S. aureus* phagosome maturation DGRP screen as a complementary approach to previous RNAi and mutagenesis screens.

We find that the cellular immune response to *S. aureus* is sexually dimorphic and that several lines of the DGRP exhibit a high degree (STD > 0.5) of phenotypic variation. These observations are in agreement with other GWA studies that looked at behavioral and physiological traits in the DGRP (Harbison et al., 2013; Swarup et al., 2013; Weber et al., 2012). Additionally, sexual dimorphism of the immune response is well documented and has been linked to the mating and fitness status of animals (Nystrand and Dowling, 2014; Short et al., 2012; Taylor and Kimbrell, 2014; Vincent and Sharp, 2014). Previous studies have assessed sexual dimorphism in the context of survival, resistance, tolerance, and induction of humoral responses following infection. Here we present compelling evidence that sexual dimorphism in Drosophila immunity extends to the cellular immune response as well. The observed sexual dimorphism in phagocytosis may underlie differences between males and females in survival and resistance to microbial infections.

Within the DGRP lines, natural polymorphisms associated with single candidate genes had the ability to affect the *S. aureus* phagosome maturation phenotype in opposite directions – either conferring an increase or decrease in phagosome maturation relative to the median for all DGRP lines. The antagonistic GWAS analysis identified multiple SNPs within single genes that conferred opposite effects within the DGRP lines. In one such instance, 10 individual intronic SNPs were identified within the gene *ypsilon schachtel (yps)* which encodes a translational regulator of mRNA localization in oocytes. Two SNPs in *yps* (3L_12116828 and 3L_12116878) were associated with increased phagosome maturation in females and decreased phagosome maturation in males (smallest *p*-value 2.61e-06). However, the eight remaining SNPs in *yps* were associated with decreased phagosome maturation in females and an increase in males (smallest *p*-value 3.23e-07). The sexually dimorphic effects of the SNPs in *yps* could be due to altered expression of *yps* in males versus females – with individual SNPs affecting transcription or splicing of *yps* in a sex-specific manner. Recently, a study to identify expression quantitative trait loci (eQTLs) in 192 DGRP lines found widespread sexual dimorphism in the gene expression patterns (Huang et al., 2015). These results lend credence to our observation of sex-specific SNP effects within the DGRP.

Interestingly, our RNAi experiments found that silencing genes in hemocytes did not always correspond to the effects of the DGRP SNPs within those loci. For example, an intronic SNP in *bifocal* was the most significant *p*-value from our screen (antagonistic *p*-value 9.4e-09). No difference in *S. aureus* phagosome maturation was observed when *bif* was silenced in hemocytes. Durham *et al*. reported similar findings after conducting a GWA using the DGRP to identify loci associated with age-related senescence and female

reproductive fitness (Durham et al., 2014). They found that the effects of SNPs within the DGRP and the effect of RNAi-mediated silencing the associated candidate genes were not always in agreement. We propose several factors that could explain the difference in results. First, the RNAi constructs used to test the effects of candidates were generated in different genetic backgrounds than the DGRP lines. Epistatic interactions between genetic variants have been implicated in starvation resistance, chill coma recovery, and startle response phenotypes within the DGRP (Huang et al., 2012). Linkage between polymorphisms in the DGRP could affect the observed *S. aureus* phagosome maturation phenotypes. The absence of the same epistatic interactions in the RNAi lines may result in the associated genes showing phenotypes different from the DGRP. Second, we used a hemocyte-specific promoter to silence the gene only in blood cells. We specifically targeted candidate genes within these cells because we were focused on how the genes affect phagocytosis and phagosome maturation of *S. aureus* within this important immune cell type. However, the SNPs identified in our GWA may have effects in other cell types and by focusing on hemocytes, we may miss these effects. By using RNAi, we only tested for loss-of-function effects and were able to characterize several novel genes whose loss-of-function in hemocytes led to phagosome maturation defects. Gain-of-function studies, particularly within hemocytes, may lead to validation of additional candidate genes from our screen.

Of the 528 candidate genes identified in our screen, 212 were novel, uncharacterized protein-coding genes and 15 were novel pseudogenes/non-protein-coding genes. We did detect variants in several genes that had previously been implicated in the immune response in the fruit fly. Genetic variants were found in *scb,* a gene that encodes

Integrin-αPS3, which, together with Integrin-βv, functions as a *S. aureus* phagocytic receptor. Variants affecting *spätzle* (*spz*), a gene encoding the Toll ligand, and *Dif*, an NFκB protein activated by the Toll receptor were also found. The Toll pathway is also important for hemocyte proliferation and density (Qiu et al., 1998; Sorrentino et al., 2002). In this capacity, the NFκB protein Dorsal (Dl), and not Dif, is the target of Toll signaling (Meister, 2004). On the other hand, during the humoral immune response, Dif is the main effector of Toll signaling and, upon infection, Dif translocates to the nucleus and induces the expression of immune-responsive transcripts such as *Drosomycin*. *Drosomycin* mRNA levels peak around 24 hours post infection (Tanji et al., 2007). However, our screen tested cellular immune responses that occur at a much earlier time point, one hour post-infection. The identification of variants in *spz* and *Dif* affecting the response at this early time point suggest an additional role for Toll signaling during phagocytosis. Thus, Toll signaling may play a role in the ability of the hemocyte to mount both cellular and humoral immune responses, an intriguing possibility that warrants further investigation.

Drosophila is a useful model to study human innate immune responses and we found orthologs of human genes associated with immunity, vesicle trafficking, cytoskeletal organization and lysosomal dynamics (Table 2). Specifically, we identified several genes with orthologs implicated in phagocytosis and the respiratory burst in neutrophils. These include *Superoxide dismutase 2* (*SOD2),* which converts superoxide ions to hydrogen peroxide to promote pathogen killing, and *Rac2* (*RAC2),* a GTPase required for activation of NOX2, the NADPH oxidase (reviewed in (Flannagan et al.,

2009)). Additionally, we identified *CG6145*, the ortholog of *NADK* (*NAD kinase*), a principal enzyme regulating cellular levels of NADPH (Lerner et al., 2001).

The screen also identified fly orthologs of human genes that mediate cytoskeletal reorganization during phagocytosis. In addition to its role during the oxidative burst, *Rac2* is an evolutionarily conserved component of the signaling cascade that controls cytoskeletal remodeling following phagocytic receptor ligation. RAC2 and Cell Division Control Protein 42 (CDC42), a Rho guanosine triphosphatase protein, are necessary for actin polymerization and pseudopod extension at the phagocytic cup (Flannagan et al., 2012). A SNP in *Zizman* (*Ziz*), the ortholog of DOCK9 (dedicator of cytokinesis 9) was also identified. DOCK9 is a guanine nucleotide exchange factor that activates CDC42 to facilitate the formation of filopodia (Meller et al., 2002). A SNP in the protein kinase *Mushroom bodies tiny* (*mbt*) was associated with *S. aureus* phagosome maturation in both males and females. The human ortholog of *mbt,* PAK2 (*P21- Protein (Cdc42/Rac) Activated Kinase 4),* is activated by Cdc42. In mouse macrophages, DOCK9 phosphorylates the myosin light chain 9 during Fcγ receptor-mediated phagocytosis (Bright and Frankel, 2011).

Furthermore, a significant SNP was identified in *Exo70*, the fly counterpart of human *exocyst complex component 7* (*EXOC7*). In Drosophila S2 cells, Exo70 promotes phagosome biogenesis and plays a role in *E. coli* uptake (Stuart et al., 2007). In murine fibroblast cells Exo70 interacts with Cdc42 to promote phagocytosis of latex beads (Mohammadi and Isberg, 2013). Finally, EXOC7 is recruited to *Salmonella typhimurium* invasion foci in HeLa cells and this recruitment facilitates bacterial invasion into the host cells (Nichols and Casanova, 2010).

During phagosome maturation the nascent, microbe-containing phagosome undergoes a series of regulated fusion events with the lysosomal compartments. Such fusion events are highly regulated and we identified SNPs in loci with human counterparts that mediate intracellular vesicle trafficking – *Vacuolar protein sorting 51* (*Vps51*)*, rugose* (*rg*)*, karst* (*kst*)*, Exo70, and Synaptotagmin12 (Syt12*). An acidic pH (~pH 4.5) in the lumen of the phagolysosome is crucial for microbial killing. At low pH, hydrolytic enzymes are activated and these enzymes digest the pathogens or particles contained within the phagolysosome. In humans, lysosomal storage disorders are caused by inherited deficiency of lysosomal enzymes that lead to lysosome malfunction. We found SNPs in loci with human counterparts implicated in lysosomal storage disorders: *CG8596*, *Lipase 4* (*Lip4*), *CG14291*, *Lysosomal α-mannosidase III* (*LManIII*), *ATP7*, and *Chitinase 2* (*Cht2*).

Candidate genes associated with *S. aureus* phagosome maturation are over-represented in Gene Ontology categories for neuronal development, ion channel activity, plasma membrane associated proteins, and proteins with IgG-like domains. Neurogenesis involves cytoskeletal remodeling and actin polymerization to facilitate cellular movement. Additionally, filopodia and lamellipodia outgrowths underlie the formation of axons and dendrites. In phagocytes, filopodia act as phagocytic tentacles and, lamellipodia form the basis of the phagocytic cup, extending over particles to form the nascent phagosome with the particle inside (Kress et al., 2007; Swanson, 2008). Eighty-three of the genes identified in our screen are associated with the generation of neurons, indicating that similar mechanisms mediate the formation and dynamics of neuronal and hemocyte outgrowths. Eleven genes implicated in potassium ion transport

across cellular membranes were also found in our screen. The role of potassium ion channels in phagocytosis has not been established and the physiological relevance of K+ during phagocytosis has been a subject of debate. Lysosomes require an acidic pH to effectively degrade internalized pathogens. While the vacuolar-type H+ ATPase pumps protons into the lumen, a secondary movement of ions from the lysosomal lumen into the cytosol is needed to dissipate the membrane potential. It has been proposed that sodium and/or potassium ions may serve as counter-ions that enable acidification (Steinberg et al., 2010). Our findings support a role for potassium ion channels; perhaps to maintain the phagosome membrane potential and ion flow during phagolysosome acidification.

Our goal was to identify novel players important for phagocytosis of *S. aureus* so we focused on potential receptors and looked for genes with loss-of-function phenotypes. Of the 38 genes tested in our RNAi experiments, three affected *S. aureus* phagosome maturation when silenced in hemocytes. There is a great deal of redundancy within the receptor repertoire for *S. aureus* in Drosophila hemocytes, and the effects of candidate genes silenced using RNAi may be masked by processes working in parallel to control the infection. The three genes verified in the RNAi screen, *dpr10*, *fred,* and *CG42673,* have not previously been shown to be involved in the immune system. Both *dpr10 and fred* are required for phagosome maturation events after *S. aureus* uptake. In contrast, the third gene, *CG42673,* is necessary for engulfment (phagocytosis) of *S. aureus* by the hemocyte.

Based on sequence similarity with the human adaptor protein, CAPON, *CG42673* is also known as Capon-like protein. Silencing of *CG42673* in hemocytes leads to decreased uptake of *S. aureus*-fluorescein labeled bioparticles, indicating that *CG42673*

is important for pathogen recognition or formation of the nascent phagosome. It is unlikely that CG42673 acts as a direct *S. aureus* receptor, as it lacks extracellular and transmembrane domains. Instead, the N-terminal phosphotyrosine-binding domain of CG42673 may be acting as a molecular scaffold to mediate the interaction between putative *S. aureus* receptors and downstream signaling molecules at the phagocytic cup. Future work to identify CG42673 binding partners in hemocytes should give insight into how CG42673 regulates *S. aureus*-specific uptake. Based on *CG42673*'s role as a cytosolic adaptor protein required for uptake of *S. aureus,* we propose the name *Staphylococcus aureus*-Receptor Adaptor Protein (StaRAP).

In Drosophila, the *fred* paralog, *echinoid* (*Ed*), is an extensively studied nectin ortholog required for the formation of epithelial cell adherens junctions (Wei et al., 2005). *Ed* regulates endocytosis of Flamingo during ommatidial development through its interaction with the adaptor protein AP-2; and, during this process, Ed localizes to Rab5 and Rab7-positive endocytic vesicles (Ho et al., 2010). Additionally, *Ed* and *fred* coordinate cellular movement during eye and wing disc sensory organ development through interactions with the Notch and EGFr signaling pathways (Chandra et al., 2003; Fetting et al., 2009). The extracellular regions of Ed and Fred share 69% identity overall, whereas the intracellular domains exhibit less similarity (30% identity) (Chandra et al., 2003). Because *Ed* and *fred* work in tandem to coordinate cellular movement during development, characterizing the phagocytosis phenotypes of *fred* and *ed* double mutants may useful. It will be interesting to see if loss of both genes confers a stronger defect in phagocytosis of *S. aureus.*

An important question concerns the mechanism by which *fred* and *dpr10* are able to modulate the maturation of *S. aureus*-containing phagosomes. Because fred and dpr10 are predicted to localize to the plasma membrane, they may recognize ligands on the bacterial surface. Alternatively, fred and dpr10 may be recruited to the phagocytic cup via interaction with other receptors that bind to *S. aureus*. The loss of these proteins does not alter uptake of *S. aureus, E. coli,* or 1μm latex beads, indicating that neither gene generically affects the cellular process of engulfment. We also found that loss of *fred* and *dpr10* affected the maturation of *S. aureus*-containing phagosome but did not alter the maturation of *E. coli*-containing phagosomes. This evidence suggests that *fred* and *dpr10* are not required for the initial uptake of *S. aureus*. Instead, their presence is required for proper intracellular trafficking or acidification of *S. aureus*-containing phagosomes. One possibility is that *fred* and *dpr10* are needed for the recruitment of endocytic machinery, such as Rab-GTPases, to the phagosome membrane. Alternatively, fred and dpr10 may be required for the formation of signaling complexes that mediate the attachment of the nascent *S. aureus*-containing phagosome to microtubules.

Phagosome autonomous maturation is the concept that the fate of an individual phagosome is dependent on the cargo within it. This idea was first proposed to describe the observation that within the same phagocyte, distinct rates of phagosome maturation can be observed – slow maturation for phagosomes containing self-ligands (i.e. apoptotic cells) versus rapid maturation of phagosomes containing Toll-like Receptor ligands (like lipid polysaccharide) (Blander and Medzhitov, 2004). Studies describing phagosome autonomous maturation have looked at self versus non-self cargo within phagosomes (reviewed in (Blander and Medzhitov, 2006)). A more recent study demonstrated a role

for JAK-STAT signaling phagosome maturation of heat-inactivated *S. aureus* in mouse macrophages (Zhu et al., 2015). In contrast to our work, this study utilized an *in vitro* cell culture system to characterize *S. aureus* phagosome maturation. Here we use an *in vivo* system to describe mutants for phagosome maturation that are specific to *S. aureus*. Our evidence supports findings that the cellular response is tailored to specific pathogens at two levels: 1) Pathogen recognition by cell surface pathogen recognition receptors. 2) Pathogen-specific phagosome maturation. In the future, it will be interesting to determine what stage of the process of phagosome maturation is altered by loss of *fred* or *dpr10* and how each of these genes drives the *S. aureus*-specific immune response.

The data presented here includes a list of over 500 candidate genes that could potentially play a role in the cellular immune response to the Gram-positive bacteria *Staphylococcus aureus.* Many of the genes are novel, and to our knowledge, have yet to be associated with the immune response in *Drosophila melanogaster*. We have found 3 new genes important for the cellular immune response. Within hemocytes, *fred, dpr10,* and *CG42673* (*StaRAP*) are required to coordinate a phagocytic response that is tailored to *S. aureus.* To our knowledge, this report is the first to show microbe-specific regulation of phagosome maturation *in vivo*. Loss of these genes perturbs the overall immune response and renders the fly less resistant to *S. aureus* infection. These findings underscore the value of Drosophila as an *in vivo* system to study the multifaceted recognition and signaling events that control the cellular immune response to *S. aureus*.

In both humans and insects, phagocytic blood cells act as the initial line of defense against *S. aureus* infections. *S. aureus* is a significant human pathogen and community-associated methicillin-resistant *S. aureus* strains pose a serious threat to

human health. A more in-depth understanding of the scope of the *Drosophila* cellular response to *S. aureus* may provide insight into the human response to *S. aureus.* The genes identified in our study provide new insight into the functional mechanisms that underlie the cellular immune response to *S. aureus* in *Drosophila melanogaster* and may provide a foundation for future work studying host-pathogen interactions and innate immunity.

V. Materials and methods

Flies and fly husbandry: DGRP stocks were generated by Dr. Trudy Mackay's laboratory at North Carolina State University. The core 40 DGRP stocks (Ayroles et al., 2009) were provided by Dr. Jeff Leips' laboratory at the University of Maryland, Baltimore County. The remaining DGRP stocks were obtained from the Bloomington Stock Center at Indiana University. w^{1118} and the blood cell-specific driver w^{1118}*; hmlΔGAL4* were from Bloomington. TRiP (http://www.flyrnai.org) RNAi lines were obtained from Bloomington and VDRC (http://www.stockcenter.vdrc.at). The *argus* mutant, and isogenic parental strain *cnbw,* were obtained from the EMS collection of Zuker lines (Koundakjian et al., 2004). Flies were reared at 25°C with 60% humidity under a 12 hour light-dark cycle and were fed a standard molasses/cornmeal/agar medium. Experiments were conducted at the same time each day.

***S. aureus in vivo* phagosome maturation screen:** We measured 12-15 three-five day-old individual flies per DGRP line using an *in vivo S. aureus* phagosome maturation assay. Female and male flies were kept in the same vial prior to being using for the experiments. To control for variability between experimental dates and conditions, we included two additional laboratory lines with each experiment: 1) *cnbw*: An isogenic

laboratory strain that is able to phagocytose *S. aureus*. 2) *argus*: A *cnbw* EMS mutant line that is unable to phagocytose *S. aureus* (unpublished). For experimental feasibility, DGRP lines were tested over the course of several days, in sets of 4-16 DGRP lines and age-matched control flies. Using a Pneumatic PicoPump PV820 (World Precision Instruments), flies were injected with pHrodo-conjugated *S. aureus* resuspended in PBS (Invitrogen: A10010, 8mg/ml). Resuspended pHrodo-*S. aureus* bioparticles were stored in small aliquots at -20°C and single aliquots were used for each set. Images of the dorsal vessel were obtained at 1 hour after injection using a Zeiss stereomicroscope (Discovery V8) with an AxioCam Hc camera. Fluorescence intensity of the area around the dorsal vessel was quantified using Axiovision 4.7 and the background fluorescence of an adjacent area was also quantified. The ratio of dorsal vessel fluorescence intensity was calculated as: $[\text{fluorescence}]_{\text{dorsal vessel area}} \div [\text{fluorescence}]_{\text{adjacent background area}}$. One-tailed *t*-tests were used to calculate *p*-values. We normalized our data by dividing the phagosome maturation phenotypes of each line and sex by the corresponding values for *cnbw* from that day.

To conduct the secondary RNA interference screen, we compared the phagosome maturation phenotypes of progeny from UAS-RNAi lines crossed to a control line w^{1118} (WT/RNAi) to the progeny of the UAS-RNAi line crossed to a *Hemolectin*-ΔGal4 line (hml>RNAi). For each genotype, we tested 12-16 control flies and 12-16 knockdown flies. A Two-tailed *t*-test was used to determine if the candidate significantly affected *S. aureus* phagosome maturation. For candidate genes that showed significant differences between control and knockdown flies during the first test, we conducted at least 2 follow-up experiments to validate the observed phenotype.

Genome-wide association analyses: To identify candidate SNPs that contribute to differences in our *S. aureus* phagosome maturation phenotype, we submitted the median fluorescence intensity phenotypes to the DGRP Freeze 2 Release 5.49 analysis pipeline (http://dgrp2.gnets.ncsu.edu/) and GWA was run using 83 of the 100 lines assayed as described in Mackay *et al.* 2012 (Mackay et al., 2012). SNPs were previously identified by whole-genome sequencing of the DGRP lines (Mackay et al., 2012). R packages qqman and ggplot2 were utilized to generate Quantile-Quantile (Q-Q) plot and Manhattan plot (Barrett et al., 2005). We initially used *p*-value threshold of $p < 10^{-5}$ for declaring SNPs to be significantly associated with the trait, but to facilitate Gene Ontology enrichment and candidate gene identification we relaxed this to $p < 10^{-4}$. False discovery rates of 0.6 (SNPs with $p < 10^{-5}$) and 0.7 (SNPs with $p < 10^{-4}$) were calculated by Dr. Lipika Ray using Bonferroni multiple testing correction. After multiple testing correction, SNPs with *p*-values < 0.05 were considered significant.

Gene Ontology analyses: Gene ontology analyses of all genes associated with SNPs in the phagosome maturation of *S. aureus* was carried out using FlyMine v41.0 (www.flymine.org) (Lyne et al., 2007).

***In vivo* phagocytosis:** To assay *S. aureus* and *E. coli* phagocytosis, approximately 6-8, 3-5 days old adults flies per genotype per experiment were injected with either fluorescein-conjugated *S. aureus* resuspended in PBS (Invitrogen: S2851, 1.6 mg/ml) or fluorescein-conjugated *E. coli* resuspended in PBS (Invitrogen: E2861, 1.6 mg/ml). Injections were performed using a Pneumatic PicoPump PV820 (World Precision Instruments). After 30 min, flies were injected again with Trypan Blue to quench extracellular fluorescence, mounted ventral side down, and images of the dorsal vessel were taken using a Zeiss

stereomicroscope (Discovery V8) with an AxioCam Hc camera. Fluorescence intensity ratios were quantified as described above.

To assay phagocytosis of beads, flies were injected with approximately 1.0μm Red Fluorescent Carboxylate Modified FluoSpheres diluted 1:20 in PBS. After 30 min, flies were injected with Trypan blue and then mounted and visualized as described above.

Survival after *S. aureus* infection: 24-30 adult flies, 5-7 days old, were injected with equal quantities of logarithmic phase culture of *S. aureus* (final OD = 0.5). Flies injected with PBS served as a wounding control. Flies were kept at 25°C, transferred regularly to new food, and death was assessed every 24 hours. The experiments were repeated at least 2 more times. Log-rank tests were used to determine if survival curves were significantly different and *p*-values <0.05 were deemed significant.

Bacteria load assays: Thirty to 50 adult flies per genotype were injected with equal quantities of logarithmic phase culture of *S. aureus* (final OD = 0.5). At 0 and 24 hours post injection, 6-8 flies from each group were immediately washed in 70% EtOH, rinsed in PBS, and homogenized in Luria-Berani media containing 1% Trition X-100. Homogenates were serially diluted and plated on Luria-Bertani agar plates. Plates were stored at 37°C overnight and colony-forming units per fly were calculated. One-tailed *t*-tests were performed and *p*-values < 0.05 were determined to be statistically significant. Experiments were done at least 3 times.

Chapter 3

A screen to identify natural polymorphisms that affect *Escherichia coli* phagocytosis in *Drosophila* hemocytes.

I. Abstract

Through the coordinated efforts of the cellular and humoral arms of innate immunity, the fruit fly is able to survive septic infections of the Gram-negative bacteria *Escherichia coli.* A subset of the *Drosophila* Genetic Reference Panel (DGRP) was screened for the ability of their hemocytes to recognize and phagocytose heat-killed, fluorescently labeled *E. coli.* The aim of the screen was to identify genes and signaling pathways that play a role in adult hemocyte phagocytosis of *E. coli*. A Genome-Wide Association Analysis (GWA) identified natural variants that were significantly associated with phagocytosis of *E. coli* within the DGRP. Overall, 18 single nucleotide polymorphisms (SNPs) were found to be significantly associated with increased phagocytosis of *E. coli.* These SNPs were located in or near ten genes. Five candidate genes were silenced using RNAi-mediated silencing in hemocytes and *E. coli* phagocytosis was assessed. RNAi of these genes in blood cells did not affect phagocytosis of *E. coli in vivo,* indicating that the SNPs may alter the function of these candidate genes in other cell types within the DGRP. An analysis of this preliminary data as well as future directions will be discussed.

II. Introduction

Escherichia coli (*E. coli*), a Gram-negative extracellular bacterium, is not generally pathogenic to the fruit fly, *Drosophila melanogaster.* However, wild type flies will succumb to infection within a few days after infection with an extremely high dose of bacteria (over $2x10^6$ bacteria cells per fly) (Shiratsuchi et al., 2014). When injected into the hemocoel of adult flies, *E. coli* Gram-negative peptidoglycan is sensed by PGRP molecules upstream of the IMD signaling pathway, inducing the activation of the NFκB transcription factor Relish (Dushay et al., 1996; Lemaitre et al., 1995).

The initial study that characterized the importance of the IMD pathway in defense against *E. coli* also made an interesting observation about a potential interaction between cellular and humoral defenses (Lemaitre et al., 1995). *imd* mutants are sensitive to *E. coli* infection, in part due to the impaired synthesis of antimicrobial peptides (AMPs) from hemocytes and fat body cells. The authors noted that *imd*/*Black cell* double mutant flies were twice as susceptible to *E. coli* than *imd* mutants alone. *Black cell* mutant larvae display a high frequency of melanotic tumors and a higher than normal hemocyte count, indicating that the *Black cell* mutation disrupts the normal development and function of crystal cells. This disrupted hemocyte niche, in conjunction with the loss of IMD signaling, was sufficient to further impair the flies ability to survive an otherwise non-lethal infection.

An elegant genetic study to define the relative roles of the humoral and cellular defense reactions during *E. coli* infections was carried out by the Schneider group (Elrod-Erickson et al., 2000). The authors carried out infection studies using a high dose of *E. coli* in *imd/imd* homozygous mutant flies. Sixty percent of *imd/imd* mutants died within

96 hours, but the flies that remained alive after 3 days were able to clear the infection. To assess the interaction between the cellular and humoral immune responses the authors demonstrated that co-injection of latex beads and bacteria inhibited the hemocytes ability to phagocytose and eliminate microbes. Co-injection of beads and *E. coli* caused 95% of *imd* homozygous mutant flies to die within the first 24 hours after infection, indicating that impairment of phagocytosis severely immunocompromised the fly.

Hemocyte genetic ablation studies have provided further lines of evidence that phagocytosis by blood cells is crucial for the *Drosophila* immune response to *E. coli.* Hemocyte-specific expression of pro-apoptotic genes, such as the murine gene *bax* or the *Drosophila* gene *reaper,* promotes hemocyte cell death via apoptosis (Defaye et al., 2009; Shiratsuchi et al., 2014). These flies die more quickly than their wild type counterparts after being injected with relatively low doses of *E. coli* (1×10^4 cells per animal). Thus, when phagocytosis has been eliminated via latex bead injections or when hemocytes have been genetically ablated, the fly becomes extremely susceptible to what would normally be a harmless *E. coli* infection. These observations indicate that microbial elimination by phagocytosis is a key component of the *Drosophila* immune response to *E. coli.*

Several phagocytic receptors for *E. coli* have been identified in cell culture studies as well as in *in vivo* genetic studies using mutant flies. To date, the known *E. coli* phagocytic PRRs are: the scavenger receptor SR-CI, the NIM-family receptors Eater and NimC1, and PGRP-LC. Dscam1 and TepII are *E. coli* opsonic receptors, but their *in vivo* immune roles, as well as the identity of their cognate receptors on the surface of

hemocytes, have yet to be defined. Additionally, the intracellular signaling complexes that form downstream from *E. coli* cell surface receptors are still unknown.

In an effort to identify genes and signaling pathways that mediate phagocytosis of *E. coli* by adult hemocytes, we carried out a small phagocytosis screen using 30 lines of the DGRP. This pilot screen identified 18 single nucleotide polymorphisms (SNPs) in or near 10 candidate genes that were significantly associated (P-value < 10^{-5}) with increased phagocytosis of *E. coli.* RNAi lines that were available at the time of our study were utilized to silence the expression of 5 candidate genes in hemocytes (*Gγ30a, CG5022, kuzbanian, iab-8, and CG16791*) and *E. coli* phagocytosis was assessed in these lines. Interestingly, hemocyte mediated silencing of the candidate genes did not affect the uptake of *E. coli.* Possible explanations for these results as well as potential future experiments will be discussed in more detail below.

III. Results and Discussion

A. An *in vivo* phagocytosis screen identifies single nucleotide polymorphisms correlated with *E. coli* phagocytosis in *Drosophila*

The *E.coli* phagocytosis assay was performed as previously described (Elrod-Erickson et al., 2000; Garg and Wu, 2014; Gonzalez et al., 2013). DGRP lines were tested over the course of several days and the mean *E. coli* phagocytosis phenotype of each line was normalized by the phenotype of an isogenic laboratory control, *cn bw.* A mutant line that shows impaired *E. coli* phagocytosis, called *argus,* was used as a negative control. The median phagocytosis value across all 30 DGRP lines was 0.904 (Figure 3.1). Student's two-tailed *t*-tests were conducted to determine if line means were

significantly different than the average for all lines. Line 324 had significantly less uptake of *E. coli* (p-value = 0.03) and line 315 showed significantly higher uptake of *E. coli* (p-value = 0.01).

DGRP mean phagocytosis phenotypes were uploaded to the Mackay laboratory's bioinformatics pipeline and GWA analyses were carried out as described in Mackay *et al.* 2012 (Mackay et al., 2012). The Mackay group utilizes phenotype data to run ANOVAs using the model: phenotype = mean + M, where M is the Marker (SNP). A total of 18 SNPs were significantly (p-value < 10^{-5}) associated with *E. coli* phagocytosis in the DGRP (Table 3.1). Interestingly, all significant associations were correlated with increased *E. coli* phagocytosis. Thus, lines carrying the minor allele for a given SNP, showed higher *E. coli* phagocytosis than lines carrying the major allele. SNP positions were determined using Flybase genome release 5.13. Significantly correlated SNPs were located in or near ten genes.

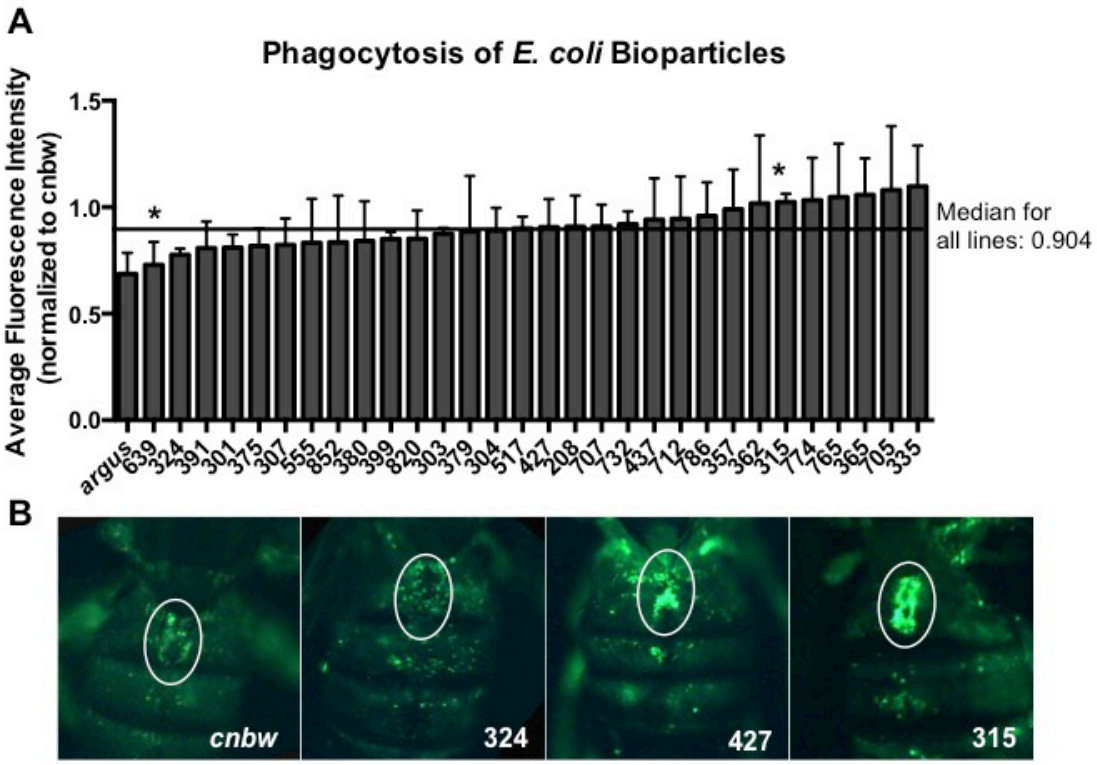

Figure 3-1: An *in vivo* phagocytosis screen to identify SNPs that affect phagocytosis of the Gram-negative bacteria *E. coli*.

(A) Phagocytosis of fluorescein-labeled *E. coli* in 30 lines of the DGRP. The average phagocytosis of each tested DGRP line was normalized to the laboratory control, *cn bw. argus,* an EMS mutant line that was previously shown to have impaired *E. coli* phagocytosis was used as a negative control. Student's two-tailed *t*-tests compared individual DGRP line means to the average across all lines. Line 324 had significantly less uptake of *E. coli* (* *p*-value = 0.03) and line 315 showed significantly higher uptake of *E. coli* (* *p*-value = 0.01). (B) Representative images from the *E. coli in vivo* phagocytosis screen. *E. coli* phagocytosis of line 427 was 0.907, and this line is representative of what the average uptake of *E. coli* within the DGRP.

SNP	Gene	SNP Site Class	*p*-value	Major allele mean	Minor allele mean	Major allele	Minor allele	Ref. allele
2L:9289970	*Gγ30A*	INTRON	5.21E-06	0.8481	0.98603	G	A	G
2L:10345609	*CG5022*	INTRON	9.42E-06	0.8740	1.02635	C	G	C
2L:10345624	*CG5022*	INTRON	9.42E-06	0.8740	1.02635	G	T	G
2L:13632604	*kuz*	INTRON	8.84E-06	0.8717	1.02465	A	C	A
2L:13632616	*kuz*	INTRON	8.84E-06	0.8717	1.02465	T	G	T
2L:16630083	*CG42389*	INTRON	7.35E-06	0.8670	1.01123	T	G	T
2R:12314225	*Sema-2b*	Synonymou	8.66E-08	0.8412	0.9980	C	T	C
3L:17926686	*Eip75B*	18,377 bp	3.45E-07	0.8535	1.0167	C	T	T
3L:17926728	*Eip75B*	18,337 bp	7.92E-07	0.8593	1.0167	C	T	T
3L:17926737	*Eip75B*	18,326 bp	7.92E-07	0.8593	1.0167	G	A	A
3L:17926835	*Eip75B*	18,228 bp	7.92E-07	0.8593	1.0167	C	A	C
3R:12626858	*abd-A*	6,491 bp	4.09E-06	0.8479	0.9913	T	C	C
3R:12626859	*abd-A*	6,490 bp	4.09E-06	0.8479	0.9913	T	C	C
3R:12683030	*iab-8*	INTRON	2.45E-06	0.8700	1.0291	C	A	C
3R:12684561	*iab-8*	INTRON	4.78E-06	0.8683	1.0272	C	G	C
3R:12685741	*iab-8*	INTRON	5.80E-07	0.8698	1.0452	G	A	G
3R:17148323	*CG16791*	INTRON	9.69E-06	0.8753	1.0347	T	G	T
3R:26586116	*zfh1*	5,532 bp	4.57E-06	0.8521	0.9914	A	C	C

Table 3-1: List of SNPs that are significantly (*p*-value<10^{-5}) associated with *E. coli* phagocytosis in the DGRP.

Single nucleotide polymorphism positions are given as Chromosome:Nucleotide position (Flybase release 5.13). MAF = Minor allele frequency of variant. Major allele mean = average *E. coli* phagocytosis phenotype of lines carrying the major allele. Minor allele mean = Average *E. coli* phagocytosis of lines carrying the minor allele. Reference allele called from the Berkeley Drosophila Genome Project reference strain y^1 cn^1 bw^1 sp^1.

B. Genes associated with *E. coli* phagocytosis in the DGRP

Table 3-1 gives the genomic positions, site class, minor allele frequency, and ANOVA derived *p*-values of the 18 top SNPs (*p*-value $<10^{-5}$) identified in the GWA analysis. Additionally, the mean *E. coli* phagocytosis phenotypes of lines carrying the major and minor alleles are given for each SNP as well as the nearest candidate genes are shown.

A SNP in *CG16791* (*p*-value = 9.69E-06) was significantly associated with *E. coli* phagocytosis in our screen. *CG16791* was previously identified as important for phagocytosis of *Candida albicans* and *E. coli* in a genome-wide S2 cell RNAi screen (Stroschein-Stevenson et al., 2006). Silencing *CG16791* in S2 cells resulted in a 50% reduction of *E. coli* and latex bead uptake and a 64% reduction of phagocytosis of *C. albicans*. Very little is known about *CG16791* and the protein contains no predicted domains. However, *CG16791* was recently identified as potential member of the *Draper*-mediated signaling pathway during phagocytosis of apoptotic cells (Fullard and Baker, 2015). Overexpression of *drpr* suppresses the development of the posterior crossvein in the wing, a phenotype that may be caused by excess apoptotic cell clearance during wing development. A chromosomal deficiency that uncovered *CG16791* as well as a *CG16791* Minos-element insertion mutant suppressed the *drpr* overexpression phenotype. Thus, CG16791 participates in a signaling pathway downstream of Draper during apoptotic cell phagocytosis.

Draper has also been implicated in phagocytosis of *E. coli in vitro.* RNAi of *Draper* in S2 cells causes a 65% reduction in the phagosome maturation of heat-killed *E. coli* bioparticles (Cuttell et al., 2008). However, due to the fact the *E. coli* particles used

in this study were labeled with the pH sensitive dye pHrodo, it is unclear if the defect caused by loss of *CG16791* was due to decreased uptake of *E. coli* or impaired phagosome maturation of *E. coli* containing phagosomes. We hypothesize that CG16791 mediates *E. coli* uptake due to the fact that a SNP in *CG16791* was significantly associated with increased uptake of *E. coli* in the DGRP lines. Thus, it is likely that *CG16791* plays a role during signaling that controls internalization of the *E. coli*, rather than the maturation of *E. coli*-containing phagosomes.

Two SNPs in *kuzbanian* (smallest *p*-value = 8.84E-06) were significantly associated with *E. coli* phagocytosis in the DGRP. Like *CG16791, kuzbanian* was identified in a whole genome S2 cell RNAi screen as important for the phagocytosis of *E. coli* and *C. albicans* (Stroschein-Stevenson et al., 2006). Kuz is a metalloprotease-disintegrin (ADAM) transmembrane protein. ADAM proteins are membrane-anchored proteases that are critical for the proteolytic cleavage and release of soluble forms of target membrane bound proteins. Kuzbanian was first described as important for nervous system development, where it functions to both promote and inhibit neural cell differentiation (Rooke et al., 1996). Kuz targets the receptor, Notch, and its ligand, Delta (Pan and Rubin, 1997; Qi et al., 1999). As a component of the Notch signaling pathway, Kuzbanian cleaves Notch to generate a functional receptor during imaginal disc development (Sotillos et al., 1997). Additionally, a screen of a collection of EMS mutagenized flies to identify genes that are important for cardiogenesis identified 5 separate EMS-induced *kuz* alleles that caused an overproliferation of cardioblast cells in the *Drosophila* heart (Albrecht et al., 2006). Hemocytes develop in larval lymph glands and the EMS-induced *kuz* mutations led to a markedly reduced number of lymph gland

cells (Albrecht et al., 2006; Jung et al., 2005). This phenotype was attributed to the fact that the loss of *kuzbanian* function led to abrogation of Notch signaling that would normally regulate development of the lymph gland. Twelve hours after pupariation, the larval lymph gland ruptures and releases differentiated hemocytes into circulation that constitute the majority of hemocytes present at the adult stage (Grigorian et al., 2011). The reduced number of lymph gland cells in *kuzbanian* mutant larvae could translate to a reduced number of hemocytes in adult flies.

A related study by the Mandal group found clusters of actively dividing hemocytes located on the dorsal side of the abdomen in *Drosophila* adults (Ghosh et al., 2015). These cells differentiate into plasmatocytes in the absence of Notch signaling or into crystal cells in the presence of Notch signaling. RNAi-mediated silencing of *Notch* in dividing adult hemocytes causes an overproliferation of phagocytic plasmatocytes. Because *kuzbanian* regulates Notch signaling in the larval lymph gland, it is possible that it may also function in a similar capacity in adult hemocyte precursor cells. Together, these studies raise the possibility that *kuzbanian* may regulate Notch signaling during hemocyte development, differentiation, or proliferation in the fly.

Our study used the *in vivo* phagocytosis assay to directly quantify fluorescence of *E. coli* bioparticles that were phagocytosed by hemocytes associated with the adult heart. If the SNPs in *kuzbanian* cause loss of function, it could lead to aberrant development of larval lymph gland cells or increased proliferation of adult plasmatocytes. The latter scenario may explain the increased *E. coli* phagocytosis observed in DGRP lines carrying the minor alleles of variants significantly associated with *E. coli* phagocytosis. Over

abundance of adult plasmatocytes in the absence of Notch signaling in adults could translate to higher levels of *E. coli* phagocytosis in the DGRP.

Another intronic SNP (*p*-value 5.21E-06) was found in the gene *Ggamma30A* (*Gγ30A*). *Gγ30A* codes the guanine nucleotide binding protein (G-protein) gamma subunit of the heterotrimeric G-protein complex which functions downstream of the G-protein coupled receptor (GPCR) rhodopsin during the phototransduction signaling cascade in the *Drosophila* eye (Schulz et al., 1999). *Gγ30A* is highly expressed in the adult head and retina but is also moderately expressed in the adult heart. Activation of Ras-signaling in hemocytes leads to sustained cell proliferation and altered gene expression (Asha et al., 2003). In Ras-activated hemocytes, Gγ30A expression was shown to increase by 6-fold. However, neither Gγ30A or alpha and beta subunits that make up the functional G-protein signaling complex, have been previously implicated in the immune response to bacteria in the fly. In mammalian macrophages, a GPCR, Brain anigiogenesis inhibitor 1 (BAI1), was recently identified as a PRR for apoptotic cells and Gram-negative, but not Gram-positive bacteria (Billings et al., 2016; Das et al., 2011).

Another SNP (*p*-value 7.35E-06) associated with *E. coli* phagocytosis in the DGRP is located in *CG42389.* CG42389 is a membrane associated protein with Fibronectin type III-like (FN3) and immunoglobulin-like (Ig-like) domains. Interestingly, many proteins that play essential roles in pathogen recognition and the immune response contain FN3 and Ig-like domains. CG42389 is an uncharacterized protein that has been shown to physically interact with falafel (flfl), a plasma membrane associated serine/threonine-protein phosphatase 4 (Lipinszki et al., 2015; Sousa-Nunes et al., 2009). RNAi of *Flfl* in S2 cells leads to a 50% reduction in the phagocytosis of *E. coli*

(Stroschein-Stevenson et al., 2006). It is possible that CG42389 acts as a cell surface PRR for *E. coli* on the surface of hemocytes and that it signals downstream through flfl to phagocytose the bacteria.

Two SNPs (*p*-value 9.42E-06) in the gene *CG5022* were associated with *E. coli* phagocytosis in the DGRP. The CG5022 protein features multiple FERM (F for 4.1 protein, E for ezrin, R for radixin and M for moesin) domains, a 3-helical bundle, and a Pleckstrin homology-like domain. FERM domain containing proteins are often involved in the localization and linkage of cytoplasmic proteins to the membrane (Chishti et al., 1998). As SNPs in CG5022 were associated with *E. coli* phagocytosis in our screen, it may possible that CG5022 serves as a scaffolding protein for the cytoplasmic signaling molecules that mediate actin cytoskeletal organization during particle uptake.

Iab-8 is a long non-coding RNA (lncRNA) that is located in the Bithorax complex between the HOX genes *abd-A* and *abd-B* (Graveley et al., 2011). Five SNPs located in or near the Bithorax complex were identified in the *E. coli* phagocytosis screen: 2 SNPs (*p*-value 4.09E-06) located ~6.5Kb downstream from *abd-A* and three SNPs (smallest *p*-value 4.09E-06) found within *iab-8*. *Iab-8* produces a microRNA (*mir-iab-8*) and a 92 Kb long non-coding RNA that both repress the expression of the homeotic gene *abd-A* during nervous system development (Gummalla et al., 2012). Abd-A plays a role in *Drosophila* heart development by establishing anterior-posterior polarity in the dorsal vessel (Lo and Frasch, 2003). It is possible that the SNPs in the Bithorax region affect the function of abd-A, which in turn alters the dorsal vessel morphology in the DGRP lines that display increased *E. coli* phagocytosis.

The most significantly associated SNP identified in the screen (*p*-value = 8.66E-08) was a synonymous coding mutation located in exon 13 of *Semaphorin-2A* (*Sema-2A*). Although extensively studied for their role in mediating axonal migration, a number of semaphorins have been implicated in the immune response in vertebrates (Roney et al., 2013). Sema-2A, is a secreted semaphorin protein with an N-terminal Sema domain and C-terminal immunoglobulin domain that mediates axon guidance in the developing brain (Bates and Whitington, 2007). The Sema domain of Sema-2A binds to the surface bound receptor, Plexin B, to regulate axon guidance in the *Drosophila* central nervous system. Interestingly, Plexin B was found in the S2 cell latex bead phagosome proteome (Stuart et al., 2007).

A single SNP (*P*-value 4.57E-06) was almost 5.5 Kb upstream from *Zn finger homeodomain 1* (*zfh1*), a transcription factor that plays an essential role in the development of plasmatocytes (Frandsen et al., 2008). *zfh1* also downregulates the IMD signaling cascade at a transcriptional level upon Gram-negative bacterial infection (Myllymaki and Ramet, 2013). Thus, the SNP upstream of *zfh1* could potentially alter the development of plasmatocytes or lead to hyperactive IMD signaling. It will be interesting to explore the effects of the SNP upstream of *zfh1* in the DGRP lines and how this relates to plasmatocyte development or IMD signaling during the immune response.

A cluster of 4 SNPs (smallest *p*-value 3.45E-07) were located ~18Kb downstream from *Ecdysone-induced protein 75B* (*Eip75B*). Eip75B is a transcriptional target of the Ecdysone nuclear receptor (EcR) and its partner Ultraspiracle (USP) (Thummel, 2001). Ecdysone is a steroid hormone that regulates developmental transitions in the fly by binding to EcR/USP dimer, which then activates the expression of primary response

genes such as the transcription factor *Eip75B*. Eip75B and other primary targets of the EcR signaling complex go on to transcriptionally activate additional genes.

Several lines of evidence indicate ecdysone signaling is important for the expression of molecules that that govern the immune response in the fly. The hemocyte derived S2 and *l(2)mbn* cell lines differentiate into macrophage-like phagocytic cells after treatment with ecdysone (Dimarcq et al., 1997). In adults, ecdysone signaling controls the expression of antimicrobial peptide genes by regulating genes that are part of the IMD pathway (Kaneko et al., 2006). In larval hemocytes, EcR signaling regulates the expression of several genes involved in phagocytosis, including the pattern recognition receptors *Nimrod, Dscam* and *PGRP-LC* (Regan et al., 2013).

Eip75B is a nuclear receptor that heterodimerizes with another ecdysone-induced nuclear receptor, DHR3, during fly metamorphosis (White et al., 1997). *Eip75B* null mutants are viable and fertile with no detectable developmental phenotypes (Bialecki et al., 2002). RNAi-mediated silencing of *Eip75B* in S2 cells and in adult flies causes a robust increase in the expression of the IMD responsive AMP genes *Attacin, Diptericin* and *Cecropin* following *E. coli* infection (Kleino et al., 2005; Rus et al., 2013). Importantly, in cell cultures and live animals, silencing of *Eip75B* causes a dramatic increase in the expression of the *E. coli* receptor, PGRP-LC (Kleino et al., 2005; Rus et al., 2013). If the SNPs downstream of *Eip75B* exert a loss-of-function effect on Eip75B, it is feasible that this could be associated with increased expression of PGRP-LC in DGRP lines carrying the SNPs. It will be necessary to experimentally assess the levels of PGRP-LC in hemocytes carrying the SNPs downstream of *Eip75B*.

C. Effects of RNAi-mediated silencing of select candidate genes in hemocytes

We obtained transgenic RNAi lines that were available through the Bloomington Stock Center to test the effects silencing candidate genes had on phagocytosis of *E. coli*. Only five of the ten candidate genes identified in our screen were available at the time of this study. We were able to test the effect silencing the following genes have on *E. coli* phagocytosis: *Gγ30A, kuz, abd-A, CG16791,* and *CG5022* (Figure 3.2). We did not observe any differences in *E. coli* phagocytosis between control flies or flies with genes of interest silenced in hemocytes.

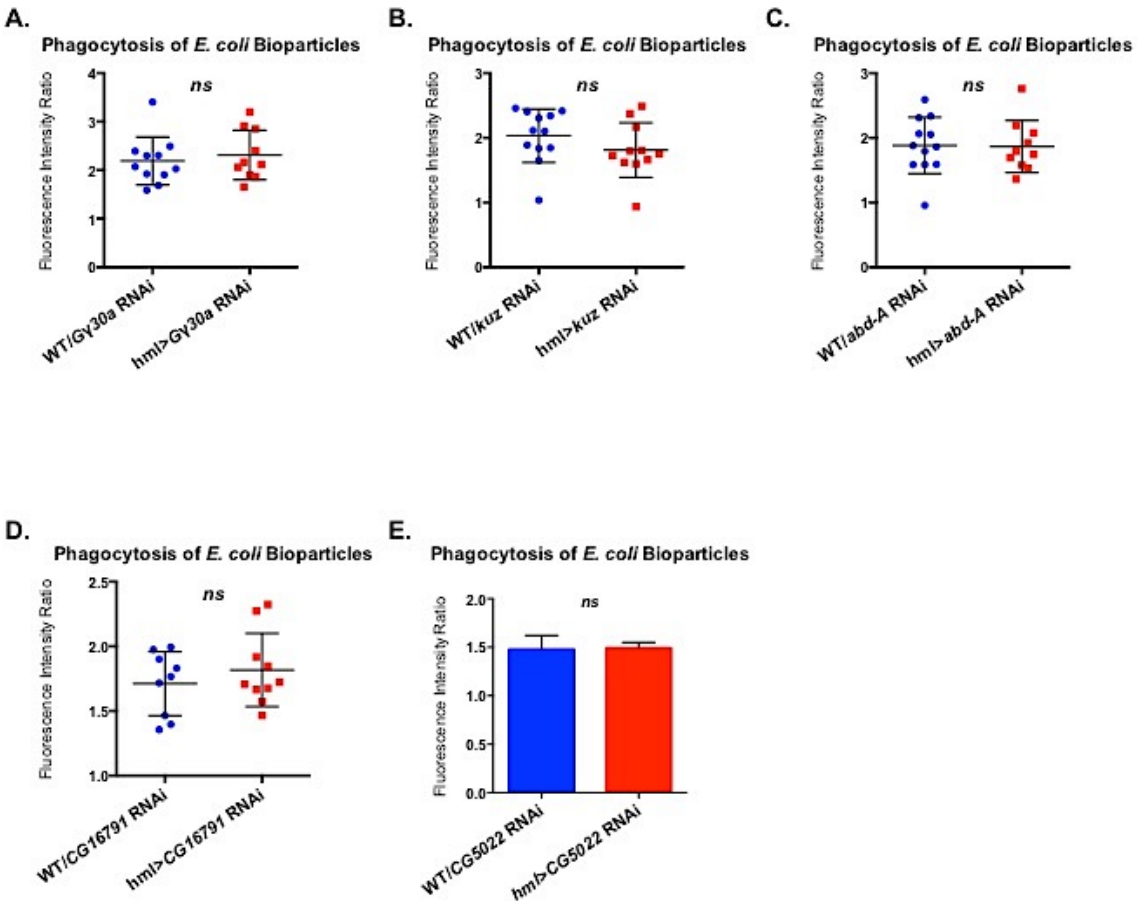

Figure 3-2: *In vivo* phagocytosis assays to test the effects of hemocyte-specific silencing of genes associated with *E. coli* phagocytosis in the DGRP.

SNPs associated with *E. coli* phagocytosis in the DGRP were located within the following candidate genes: *Gγ30a, kuzbanian, CG61791* and *CG5022. Abdominal-A* RNAi was tested due to the fact that three SNPs were located within the long-non-coding RNA, *iab-8*, which in turn regulates *abdominal-A.* To silence genes in hemocytes, gene-specific TRiP RNAi lines were crossed to the hemocyte-driver, *hmlΔGAL4.* As a control, TRiP lines were crossed to the *hmlΔGAL4* background line, w^{1118} (WT). Ten-to-12 progeny from each cross were tested once: (A) *Gγ30a,* (B) *kuzbanian,* (C) *abd-A,* (D) *CG16791* or three times: (E) *CG5022*. Statistical analysis: Two-tailed *t*-tests. Error bars = SEM.

Phagocytosis is a highly complex process that involves simultaneous pathogen recognition by multiple different receptors to initiate signaling cascades that lead to particle internalization. Before going any further with the genetic analyses of the cellular immune roles of genes identified in this screen, an analysis how SNPs affect gene expression is in order. Prior to carrying out any additional screens, qPCR should be used to compare the expression levels of genes of interest in DGRP lines carrying the minor alleles versus those carrying the major alleles. This analysis could also determine if the SNPs lead to increased or decreased mRNA expression of candidate genes or if the effects of the SNPs occur at the level of post-transcriptional regulation.

It is possible that the candidate genes identified in our screen participate in parallel signaling cascades initiated from different *E. coli* phagocytosis receptors. Thus, the combined effects of multiple SNPs in the DGRP would be required to bring about the increased level of *E. coli* phagocytosis that several lines of the DGRP displayed. To address this possibility, it will be helpful to create transheterozygote flies that express 2 or more RNAi transgenes against genes of interest in hemocytes. An alternative approach may be to simultaneously inject dsRNA constructs targeting multiple genes to silence the expression of several genes of interest at once.

Finally, it will be worthwhile to test all genes associated with the SNPs identified in this GWA. A literature search of the candidates showed that the genes could be potentially affect the immune response through several mechanisms: (A) Mediate heart development (kuz, iab-8, and abd-A); (B) Mediate hemocyte development (kuz, Eip75B or zfh1); (C) Regulate the expression of genes that play a role in *E. coli* recognition (zfh1 or Eip75B); (D) Regulate actin cytoskeletal dynamics at the phagocytic cup (CG5022) or

(E) Be directly involve in pathogen recognition or signaling from *E. coli* cell surface receptors (CG16791, CG42389, Sema-2A, or Gγ30A).

The work described herein may lay the foundation of future projects to study novel interactions between *Drosophila* hemocytes and *E. coli.* Hemocytes are vital to protect the fruit fly from septic infections with *E. coli.* Mutant flies lacking the capacity to mount humoral defenses succumb to *E. coli* infections about 50% of the time. This susceptibility dramatically increases when phagocytic capacity of these same flies is inhibited, illustrating the importance of cooperation between hemocytes and antimicrobial peptides during infection in the fly. Hemocyte recognition, phagocytosis, and degradation of *E. coli* are needed to control the growth of *E. coli* within the fly.

IV. Materials and Methods

Flies and fly husbandry: DGRP stocks were generated by Dr. Trudy Mackay's laboratory at North Carolina State University. The core 40 DGRP stocks (Ayroles et al., 2009) were provided by Dr. Jeff Leips' laboratory at the University of Maryland, Baltimore County. The remaining DGRP stocks were obtained from the Bloomington Stock Center at Indiana University. w^{1118} and the blood cell-specific driver w^{1118}; *hmlΔGAL4* were from Bloomington. TRiP (http://www.flyrnai.org) RNAi lines were obtained from Bloomington. To test the effects of silencing candidate genes in hemocytes, RNAi lines were crossed to *hmlΔGAL4* flies or to w^{1118} as a control The *argus* mutant, and isogenic parental strain *cnbw,* were obtained from the EMS collection of Zuker lines (Koundakjian et al., 2004). Flies were reared at 25°C with 60% humidity under a 12 hour light-dark cycle and were fed a standard molasses/cornmeal/agar medium. Experiments were conducted at the same time each day.

***In vivo* phagocytosis:** To assay *E. coli* phagocytosis, approximately 6-8, 3-5 days old adults flies per genotype per experiment were injected with fluorescein-conjugated *E. coli* resuspended in PBS (Invitrogen: E2861, 1.6 mg/ml). Injections were performed using a Pneumatic PicoPump PV820 (World Precision Instruments). After 30 min, flies were injected again with Trypan Blue to quench extracellular fluorescence, mounted ventral side down, and images of the dorsal vessel were taken using a Zeiss stereomicroscope (Discovery V8) with an AxioCam Hc camera. Fluorescence intensity ratios were quantified as described above. Experiments were performed at least three times for each genotype tested.

Genome-wide association analysis: To identify candidate SNPs that contribute to differences in *S. aureus* phagocytosis, we tested 30 lines of the DGRP using the *in vivo* *E. coli* phagocytosis assay. We submitted the median fluorescence intensity phenotypes to the DGRP Freeze 1 Release 5.49 analysis pipeline (http://dgrp.gnets.ncsu.edu/) and GWA was performed as described in Mackay *et al.* 2012 (Mackay et al., 2012). SNPs were previously identified by whole-genome sequencing of the DGRP lines (Mackay et al., 2012) and SNP positions were annotated according to Flybase Release 5.49.

Chapter 4

The RNA splicing factor Ataxin-2 binding protein 1 is required for the cellular immune response in Drosophila phagocytes.

I. Abstract

To identify novel genes and signaling pathways involved in phagocytosis of bacteria, we screened a subset of the Drosophila Genetic Reference Panel (DGRP) for the ability of their hemocytes to phagocytose the Gram-positive bacteria *Staphylococcus aureus*, a major human pathogen (Mackay et al., 2012; Miller and Cho, 2011; van Kessel et al., 2014). One of the genes identified by our screen was *Ataxin 2 Binding Protein-1* (*A2bp1*). *A2bp1* is a member of the highly conserved Fox-1 family of RNA-binding proteins (Bajpai et al., 2004; Kuroyanagi, 2009; Shibata et al., 2000). Fox-1 family members regulate tissue-specific alternative splicing by binding to a (U)GCAUG element in regulated exons or flanking introns of mRNA precursors (Auweter et al., 2006; Fukumura et al., 2007; Jin et al., 2003; Ponthier et al., 2006; Underwood et al., 2005). Human *A2bp1* orthologs, also known as *RBFOX 1, 2, and 3* have been linked to brain development, cardiac function, and Autism Spectrum Disorders (Bhalla et al., 2004; Bill et al., 2013; Gao et al., 2015; Lovci et al., 2013; Martin et al., 2007; Shibata et al., 2000; Voineagu et al., 2011; Weyn-Vanhentenryck et al., 2014; Zhang et al., 2008). In *Drosophila, A2bp1* mediates a diverse range of developmental and cellular processes (Bajpai et al., 2004; Jordan et al., 2012; Tastan et al., 2010; Usha and Shashidhara, 2010). A number of RNA-binding proteins regulate mRNA stability, splicing and post-

transcriptional responses during mammalian and insect immune responses (Dong et al., 2012; Kafasla et al., 2014; Riddell et al., 2014). Flies with *A2bp1* silenced or overexpressed in phagocytic blood cells (hemocytes) are specifically impaired in phagocytosis and survival following *S. aureus* infection. To identify A2bp1 targets, we performed transcriptome analysis in isolated adult hemocytes. Twenty genes were differentially expressed in hemocytes when *A2bp1* expression was silenced via RNAi. An additional 25 genes were differentially expressed after *S. aureus* infection. One of the genes negatively regulated by A2bp1 in adult blood cells is the Immunoglobulin-superfamily member *Down syndrome adhesion molecule 4* (*Dscam4*). RNAi-mediated silencing of *A2bp1* and *Dscam4* in blood cells rescued the fly's immune response to *S. aureus* indicating that Dscam4 negatively regulates *S. aureus* phagocytosis.

II. Results and Discussion

A. A SNP in *A2bp1* is associated with reduced phagocytosis of *Staphylococcus aureus*

In order to characterize natural genetic variation in the innate immune response of *Drosophila* to Gram-positive (*S. aureus*) bacteria, we screened 30 lines of the DGRP using a *in vivo* adult phagocytosis assay (Figure 4-1A) (Elrod-Erickson et al., 2000). To control for experimental variability, the data was normalized to an isogenic laboratory strain, *cn bw*. A previously characterized line known to have defects in *S. aureus* phagocytosis (*argus*), served as a negative control. The median phagocytosis for all 30 DGRP lines was 0.99. Phagocytosis images are shown for line 365 (showing median phagocytosis), line 307 (with significantly reduced phagocytosis) and line 786 (with significantly higher phagocytosis) (Figure 4-1A). A schematic of how the fly is imaged in

the phagocytosis assay is shown in Figure 4-1B. Genotype-phenotype association analyses revealed that a SNP in the second intron of *A2bp1* (minor allele ***(A)*** at position 3L:10538501, *p-value* 6.67 E-06) was a natural polymorphism found in lines with reduced phagocytosis of *S. aureus* bioparticles, such as line 307 (Figure 4-1A and C).

Eight splice forms are produced from *A2bp1* (Figure 4-1C). The transcription of two isoforms, *A2bp1-RL* and *A2bp1-RH,* is initiated 34.5kB upstream of the remaining six isoforms. The 5'UTR and first and second exons included in *A2bp1-RL* and *A2bp1-RH* are unique to these transcripts. *A2bp1-RE, -RF, -RJ, -RI, -RK,* and *–RM* share the same transcription start site and a common 5'UTR sequence. However, all of the isoforms contain common exons that are translated into the highly conserved Fox 1-family RNA-recognition motif.

We verified that *A2bp1* is expressed in adult hemocytes by quantifying the mRNA levels of *A2bp1* in GFP-positive adult hemocytes sorted by fluorescence-activated cell sorting (Figure 4-2A). We then compared the expression levels of *A2bp1* in DGRP lines with the major allele ***(T)*** at 3L:10538501 to lines with the minor allele ***(A)*** at position 3L:10538501. *A2bp1* mRNA levels were significantly reduced in larval hemocytes from line 307 compared to hemocytes from line 365 (Figure 4-2B). In adult flies, expression of two isoforms of *A2bp1* (*A2bp1-RL* and *A2bp1-RH*) was significantly reduced in lines carrying the ***(A)*** at 3L:10538501 (Figure 3-2C). These results indicate that ***(A)*** at 3L:10538501 correlates with decreased expression of specific isoforms of *A2bp1* in the affected DGRP lines.

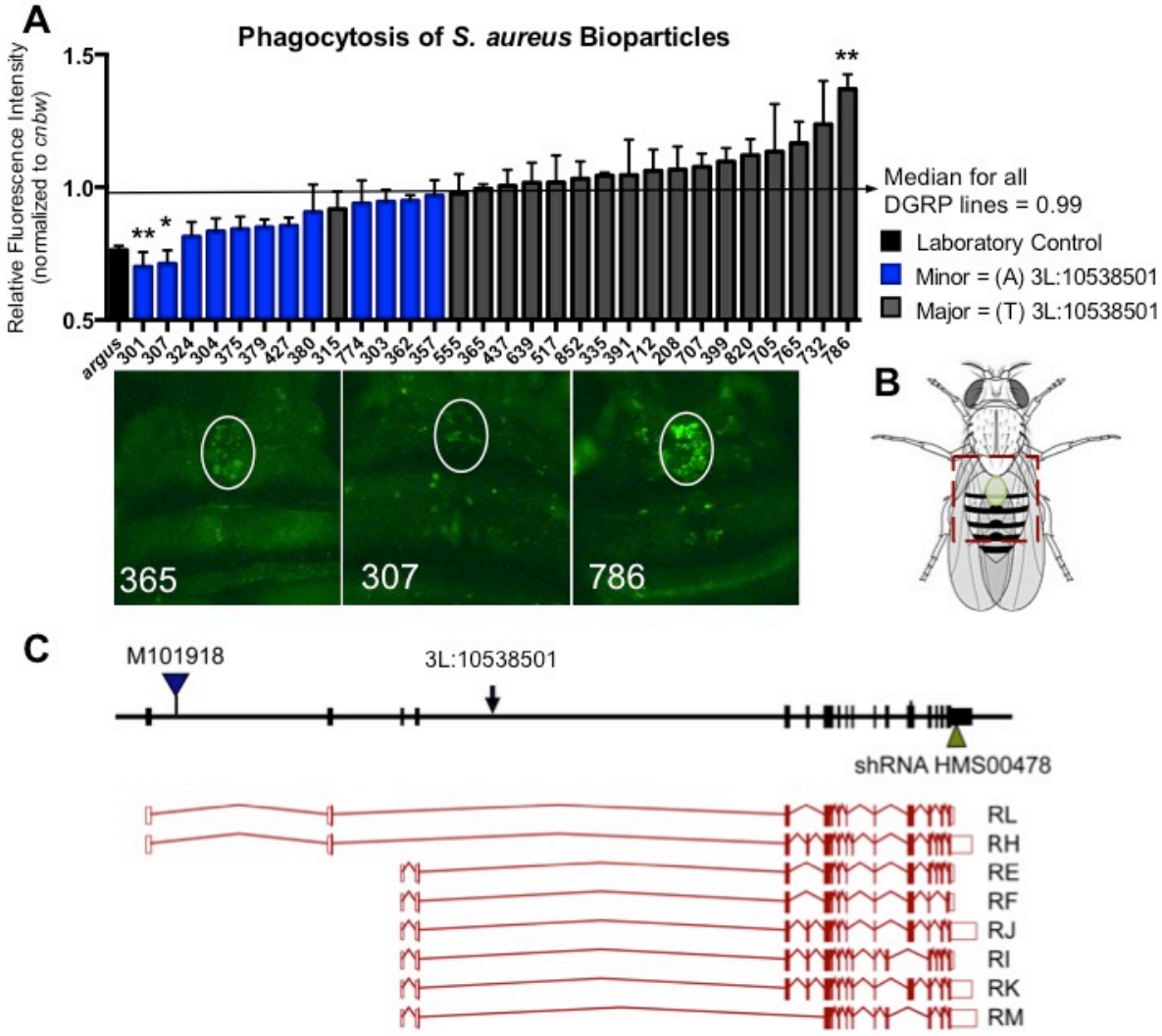

Figure 4-1: An *in vivo* phagocytosis screen identifies a SNP in *A2bp1* that affects the uptake of *S. aureus*.

(A) Phagocytosis of fluorescein-labeled *S. aureus* in 30 lines of the DGRP. (B) Depiction of how the fly is visualized during the adult phagocytosis assay. (C) Schematic showing the genomic region of *A2bp1* (black bars) and the 8 isoforms expressed from the gene (red bars). Location of SNP 3L:10538501 indicated with black arrow. The blue, inverted triangle shows where the Minos transposon M101918 is located within *A2bp1*. The TRiP short hairpin RNA construct HMS00478 targets a 21 base pair sequence in the 3'UTR of all isoforms; depicted by the green triangle. Error bars, ± SEM. * *p*-value<0.05, ** *p*-value<0.01, ns = not significantly different.

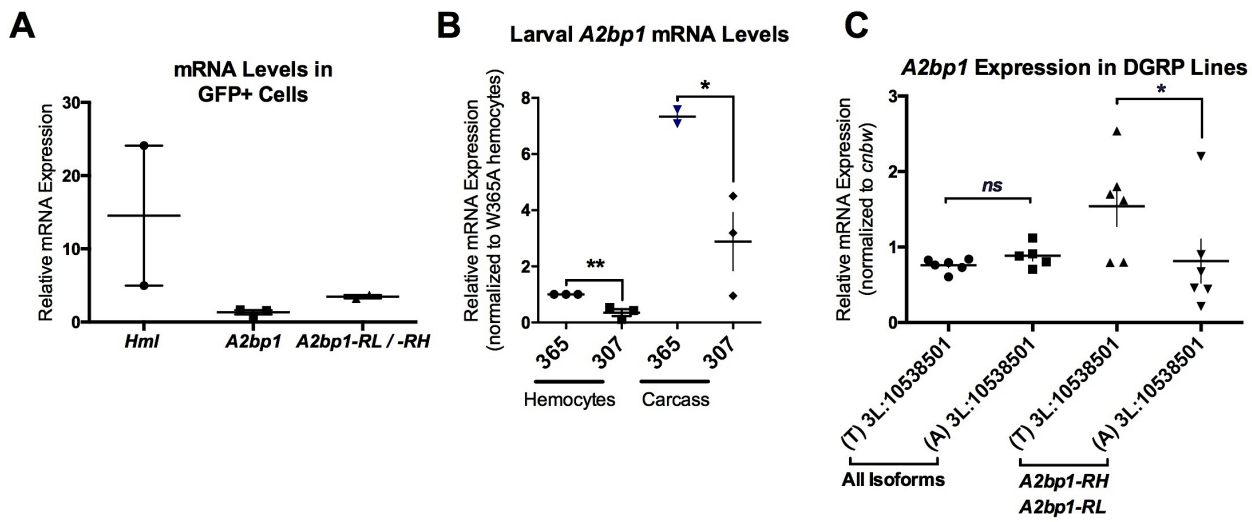

Figure 4-2: Quantitative PCR analysis of the expression of *A2bp1* in *Drosophila*.

(A) Expression of *Hemolectin* (*hml*), *A2bp1,* and *A2bp1* isoforms *–RL* and *–RH* in FACs sorted GFP- positive adult hemocytes. (B) Expression of *A2bp1* in larval hemocytes and carcasses from lines 365 and 307. Hemocytes were collected from 40 larvae. (C) Expression of *A2bp1* and of *A2bp1* isoforms *–RL* and *–RH* in DGRP lines. Six flies were pooled from each DGRP line. n= 6 DGRP lines with the major allele ***(T)*** (787, 208, 365, 437, 315, and 555) and n=6 DGRP lines with the minor allele ***(A)*** (239, 307, 320, 149, 158, and 362) at position 3L:10538501. Transcript levels were measured via qPCR and *rp49* was used as an endogenous control. Experiments were performed in triplicate. Error bars, ± SEM. * p-value<0.05, ns = not significantly different.

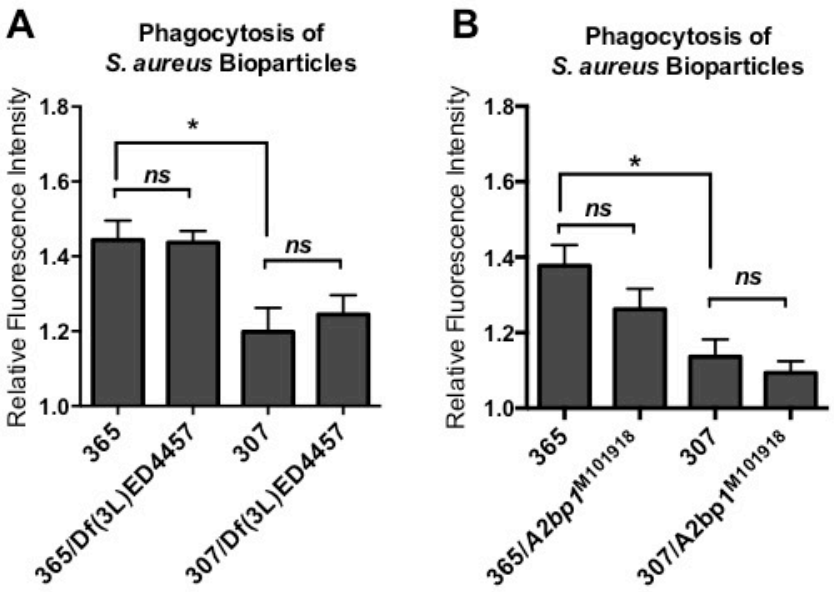

Figure 4-3: Deficiency and Transposon insertion complementation tests confirm that a SNP in *A2bp1* leads to impaired *S. aureus* phagocytosis.

(A) Phagocytosis of fluorescein-labeled *S. aureus* in lines 365 and 307 crossed to a chromosomal deletion line (3L(ED4457)) that uncovers the genomic region of *A2bp1*. (B) Phagocytosis of fluorescein-labeled *S. aureus* in lines 365 and 307 crossed to a line carrying a transposon insertion that disrupts the expression of *A2bp1,* Mi[MIC]*A2bp1*^{M101918}. For adult phagocytosis assays, 6-8 flies per genotype were used in each experiment. Experiments were performed in triplicate. Error bars, ± SEM. * *p*-value<0.05, ** *p*-value<0.01, ns = not significantly different.

To see if ***(A)*** at 3L:10538501 exerts its effect through a loss-of-function, we performed deficiency and transposon complementation tests. A deficiency (Df(3L)ED4457) uncovering the genomic region of *A2bp1* (Chromosomal deletion from 67E2 to 68A7) failed to complement DGRP line 307. Progeny from this cross exhibited impaired *S. aureus* phagocytosis compared to control files, DGRP line 365/ Df(3L)ED4457 (Figure 4-3A). Additionally, a transposon insertion line Mi[MIC]*A2bp1*^{M101918} also failed to complement line 307, when compared to control flies (Figure 4-3B). These results indicate that the SNP in *A2bp1* causes a loss-of-function defect.

A transposon insertion in *A2bp1* impairs the cellular immune response to *S. aureus*

Phenotypic analysis of homozygous *A2bp1*^{M101918} mutants revealed that these flies showed *S. aureus*-specific phagocytosis defects and altered the expression of *A2bp1* transcripts (Figure 4-4A-C). The *A2bp1*^{M101918} insertion caused an increase in expression of the first and second exons common to all isoforms, but a decrease in expression of *A2bp1-RL* and *A2bp1-RH* in blood cells (Figure 4-4D). To examine if the transposon insertion in *A2bp1* impairs the flies ability to fight an infection with *S. aureus,* we injected log-phase *S. aureus* (OD 0.2) into the abdomen of adult flies. *A2bp1*^{M101918} flies were more susceptible to *S. aureus* infection than the background control, w^{1118} (Figure 4-4E). Because the *A2bp1*^{M101918} insertion differential altered the expression of *A2bp1* isoforms, it was unclear whether decrease or increase of *A2bp1* isoform expression led to the immune dysfunction.

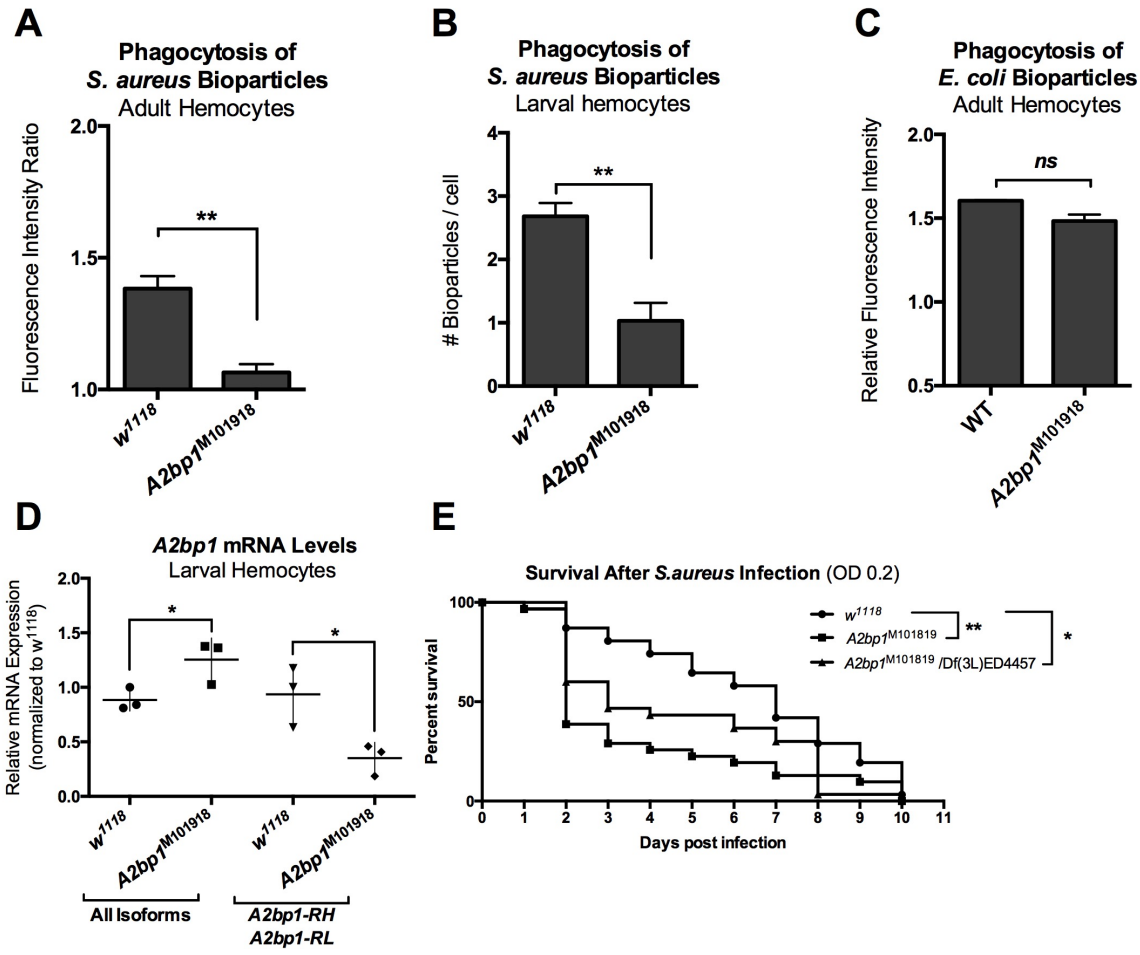

Figure 4-4: A transposon insertion in *A2bp1* impairs the immune response to *S. aureus*.

(A) Phagocytosis of fluorescein-labeled *S. aureus* bioparticles in w^{1118} *and* $A2bp1^{M101918}$ flies. (B) Phagocytosis of fluorescein-labeled *S. aureus* by hemocytes from w^{1118} and $A2bp1^{M101918}$ larvae. (C) Phagocytosis of fluorescein-labeled *E.coli* bioparticles in w^{1118} *and* $A2bp1^{M101918}$ flies. (D) Expression of *A2bp1* in hemocytes from w^{1118} *and* $A2bp1^{M101918}$ larvae. (E) Representative survival curve of w^{1118}, $A2bp1^{M101918}$, $A2bp1^{M101918}$/Df(3L)ED4457 flies after injection with *S. aureus* (OD 0.5) n = 28-30 flies. Log-rank (Mantel-cox) tests were used to determine of survival after infection was significantly different between the tested lines. All experiments were performed at least in triplicate. Error bars, ± SEM. * p-value<0.05, ** p-value<0.01, ns = not significantly different.

B. Altering the expression of *A2bp1* in blood cells specifically alters the immune response to *S. aureus*.

To determine whether increased or decreased expression of *A2bp1* led to impaired *S. aureus* uptake, we directly manipulated *A2bp1* levels in hemocytes. We expressed two separate *A2bp1* RNAi constructs specifically in blood cells using the *hml*ΔGAL4 driver. Phagocytosis of *S. aureus* bioparticles was significantly decreased in hmlΔ>*A2bp1* RNAi larval and adult hemocytes as compared to control flies (Figure 4-5A and B). Phagocytosis of live, GFP-expressing *S. aureus* was also significantly decreased in hmlΔ>*A2bp1* RNAi flies (Figure 4-5C). The *A2bp1* RNAi construct HMS00478 is a short hairpin RNA that targets a 21 base pair sequence in the 3'UTR of all isoforms and all additional RNAi experiments were carried out using this line.

Expression of a transgenic *A2bp1-RE* in the *A2bp1* RNAi flies was sufficient to rescue the *S. aureus* phagocytosis phenotype (Figure 4-5A and B). However, overexpression of *A2bp1-RE* in a wildtype background led to dramatically reduced phagocytosis of *S. aureus* (Figure 4-5D). Of note, hmlΔ>*A2pb1* RNAi and hmlΔ>*A2bp1-RE* flies do not have fewer blood cells and show no changes in the phagocytosis of *E. coli* and latex beads (Figure 4-6). This indicates that the blood cells of these flies develop normally, posess functional phagocytic machinery, but are specifically unable to phagocytose *S. aureus.* Based on these results, we concluded that the expression level of *A2bp1* must be tightly regulated in hemocytes for an effective cellular immune response to *S. aureus.*

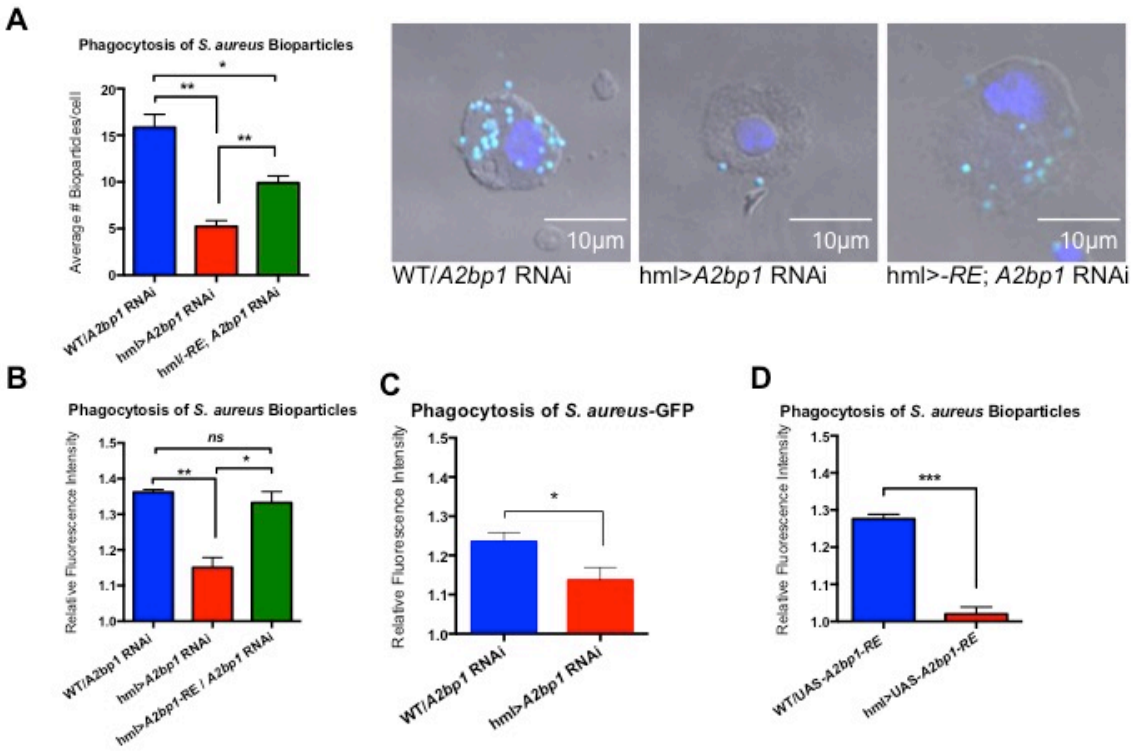

Figure 4-5: *S. aureus* phagocytosis is affected when *A2bp1* expression is either increased or decreased in hemocytes.

(A) Quantification and representative images of phagocytosis of fluorescein-labeled *S. aureus* by larval hemocytes from in WT/*A2bp1* RNAi, hmlΔ>*A2bp1* RNAi and hmlΔ>*A2bp1-RE/A2bp1* RNAi flies. For each experiment, ten larvae were injected with equal amounts of fluorescein-labeled *S. aureus*. Approximately 10 cells per larvae were imaged and individual bioparticles per cell were counted. Blue = DAPI, Green = fluorescein-labeled *S. aureus,* DIC to visualize cell boundary. (B) Adult phagocytosis of fluorescein-labeled *S. aureus* in WT/*A2bp1* RNAi, hmlΔ>*A2bp1* RNAi and hmlΔ>*A2bp1-RE/A2bp1* RNAi flies. (C) Quantification of phagocytosis of live *S. aureus* expressing GFP (OD 5.0) in WT/*A2bp1* RNAi and hmlΔ>*A2bp1* RNAi flies. (D) Adult phagocytosis of fluorescein-labeled *S. aureus* in WT/*A2bp1-RE* and hmlΔ>*A2b1-RE* flies. For adult phagocytosis assays, 6-8 flies were tested in each experiment. All experiments were performed at least three times. Error bars, ± SEM. * *p*-value<0.05, ** *p*-value<0.01, *** value<0.001, *** value<0.0001, ns = not significantly different.

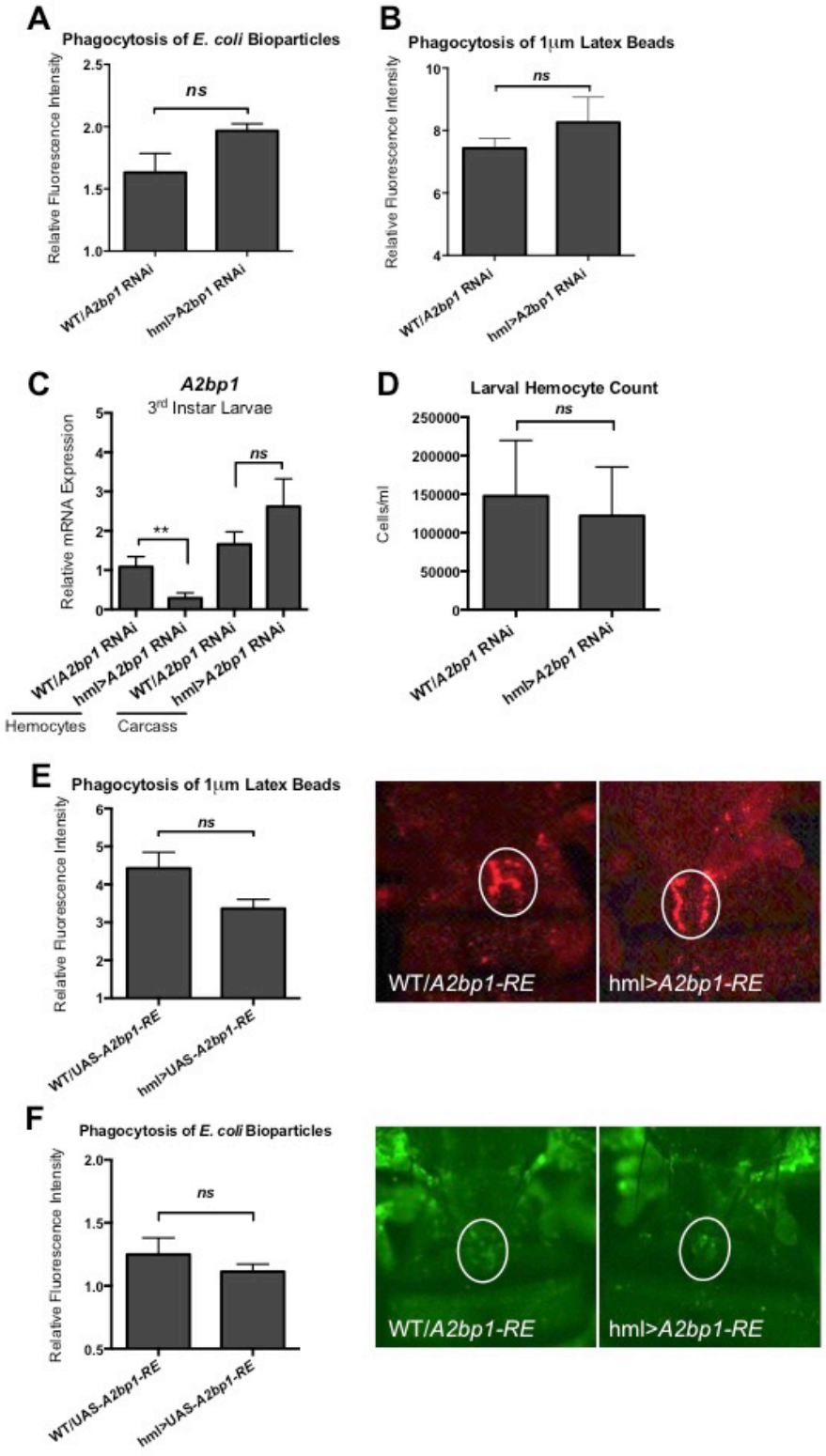

Figure 4-6: Altered *A2bp1* expression in hemocytes has no effect on hemocyte development or phagocytosis of latex beads or *E. coli*.

Figure 4-6: Altered *A2bp1* expression in hemocytes has no effect on hemocyte development or phagocytosis of latex beads and *E. coli*.

(A) Phagocytosis of fluorescein-labeled *E. coli* in WT/*A2bp1* RNAi and hmlΔ>*A2bp1* RNAi flies. (B) Quantification of phagocytosis of 1μM red fluorescent latex beads WT/*A2bp1* RNAi and hmlΔ>*A2bp1* RNAi flies. The experiment was performed three times. (C) Comparison of *A2bp1* mRNA levels in hemocytes and carcasses from WT/*A2bp1* RNAi and hmlΔ>*A2bp1* RNAi flies. 40 larvae of each genotype were pooled for each experiment. (D) Comparison of the in WT/*A2bp1* RNAi and hmlΔ>*A2bp1* RNAi larvae. Hemocytes bled into PBS and counted using a hemocytometer. Ten larvae per genotype were used. (E) Phagocytosis of 1μM red fluorescent latex beads WT/*A2bp1-RE* and hmlΔ>*A2bp1-RE* flies. (F) Phagocytosis of fluorescein-labeled *E. coli* in WT/*A2bp1-RE* and hmlΔ>*A2bp1-RE* flies. Approximately six flies per genotype were used in each adult *in vivo* phagocytosis experiment and all experiments were performed in triplicate. Error bars, ± SEM. * p-value<0.05, ns = not significantly different.

Blood-cell specific silencing or overexpression of *A2bp1* also caused increased susceptibility to *S. aureus* infection (Figure 4-7A and B). Hence, we were unable to rescue the susceptiblity phenotype of *A2bp1* RNAi flies by co-expressing *A2bp1-RE* in hemocytes (Figure 4-7A). It is possible that in addition to its role during the immediate phagocytic response, A2bp1 may regulate post-transcriptional splicing events at later time points during *S. aureus* infections. Both hmlΔ>*A2bp1* RNAi and hmlΔ>*A2bp1-RE* flies showed an increased bacteria load following *S. aureus* infection (Figure 4-7C and D). Thus, altered *A2bp1* expression directly affects the fly's ability to limit the growth of bacteria, and this may be due to misregulated expression of A2bp1 targets.

The impaired immune response of the $A2bp1^{M101918}$ mutant was specific to *S. aureus*. Therefore, we assessed if silencing or overexpressing *A2bp1* in hemocytes also altered the the cellular immune response in a *S. aureus* specific manner. RNAi mediated silencing of *A2bp1* in hemocytes did not affect the uptake of fluorescein-labeled *E. coli* or latex beads (Figure 4-6A and B). Additionally, when *A2bp1* was silenced in larval hemocytes, it did not affect hemocyte development of blood cells (Figure 4-6C and D). Similarly, hemocyte- specific overexpression of UAS-*A2bp1-RE* did not affect phagocytosis of *E. coli* or latex beads. Together, these findings reveal that altered expression of *A2bp1* does not cause general defects in the phagocytic machinery of fly hemocytes.

C. Loss of *A2bp1* does not change the immune response to the Gram-positive bacteria *Listeria monocytogenes*

Wild-type fruit flies will succumb to infections of low doses of the intracellular bacteria *Listeria monocytogenes* within a week of infection (Mansfield et al., 2003).

Homozygous *A2bp1*^{M101918} flies and flies expressing *A2bp1*-RNAi in hemocytes were not more susceptible to infection with *L. monocytogenes* (Figure 4-8A-B). Thus, the loss of *A2bp1* does lead to general defects in survival against Gram-positive pathogens. Rather, our evidence suggests that loss of *A2bp1* specifically alters the immune response to *S. aureus*. Due to the specificity of the impaired immune response, we reasoned that A2bp1 may be important the post-translational processing of genes that are important for *S. aureus* recognition or uptake.

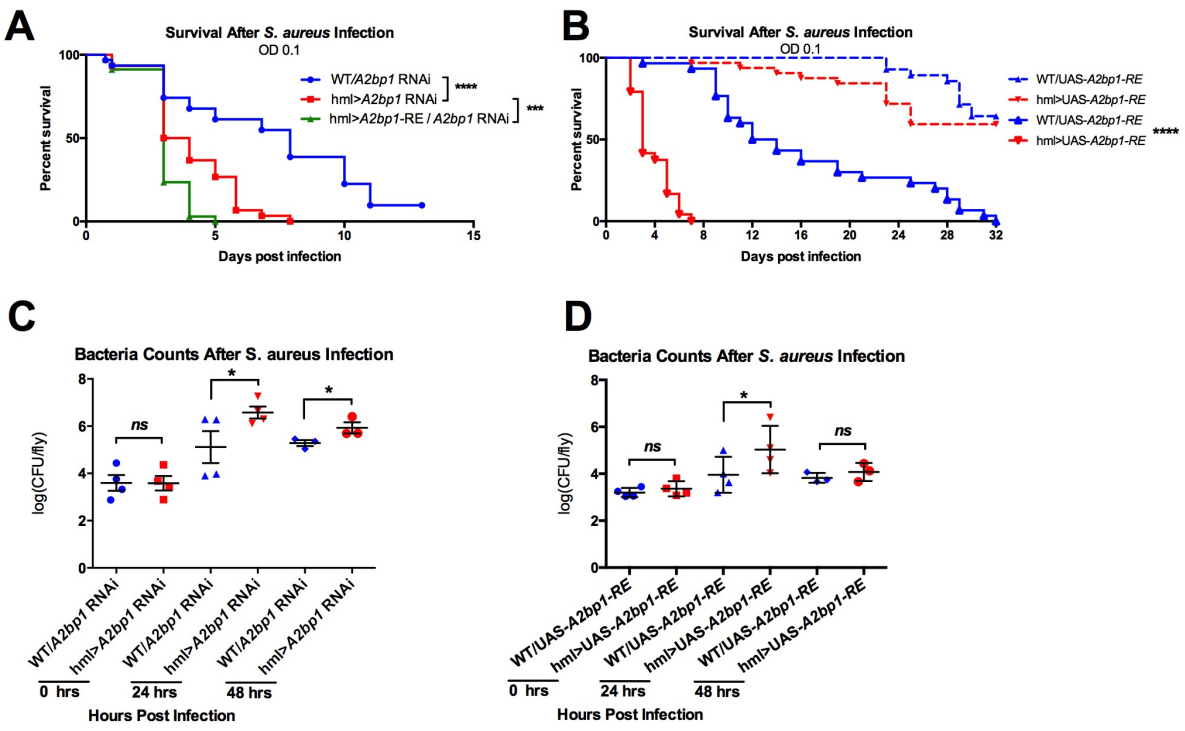

Figure 4-7: Adult fly survival and resistance after *S. aureus* infection are affected when *A2bp1* is either increased or decreased in hemocytes.

(A) Representative survival curve of WT/*A2bp1* RNAi, hmlΔ>*A2bp1* RNAi and hmlΔ>*A2bp1-RE/A2bp1* RNAi flies after injection with *S. aureus* (OD 0.1) n = 24-30 flies. (B) Representative survival curve of WT/*A2bp1-RE* and hmlΔ>*A2b1-RE* flies after injection with *S. aureus* (OD 0.1) n = 24-30 flies. (C) *S. aureus* (OD 0.5) in WT/*A2bp1* RNAi and hmlΔ>*A2bp1* RNAi at 0, 24, and 48 hours post infection. (D) Comparison of *S. aureus* (OD 0.5) recovered in WT/*A2bp1-RE* and hmlΔ>*A2bp1-RE* at 0, 24, and 48 hours post infection. Bacteria load was measured in six to eight individual flies per genotype at each time point in each experiment. Experiments were performed at least 3 times and the mean bacteria load is shown for each experiment. For adult phagocytosis assays, 6-8 flies were tested in each experiment. All experiments were performed at least three times. Error bars, ± SEM. * p-value<0.05, ** p-value<0.01, *** value<0.001, *** value<0.0001, ns = not significantly different.

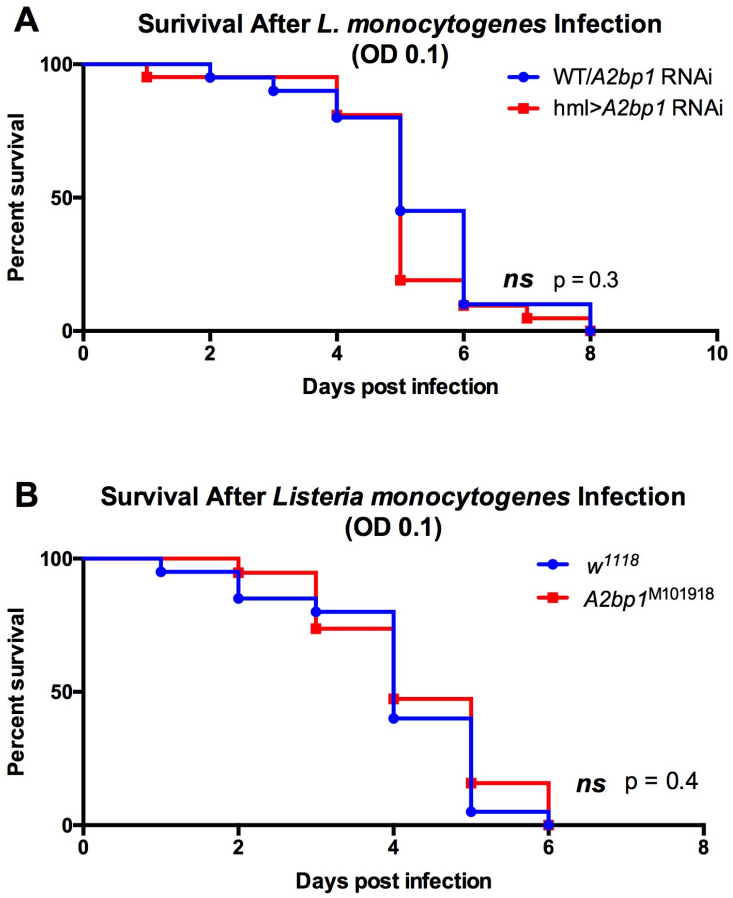

Figure 4-8: *A2bp1* mutant and RNAi flies are not more susceptible to *L. monocytogenes* infection than control flies.

(A) Representative survival curve of w^{1118} and $A2bp1^{M101918}$ flies after injection with *L. monocytogenes* (OD 0.1) n = 24-30 flies. (B) Representative survival curve of WT/*A2bp1* RNAi and hmlΔ>*A2bp1* RNAi flies after injection with *L. monocytogenes* (OD 0.1) n = 24-30 flies. All experiments were performed at least three times. ns = not significantly different.

D. Transcriptome analysis of wild-type and *A2bp1*-RNAi adult hemocytes

We reasoned that *A2bp1*, as a splicing factor, is likely to be regulating post-transcriptional processing of mRNAs that are important for managing the host response against *S. aureus*. To identify transcripts that are affected in adult hemocytes after loss of *A2bp1*, we utilized RNA sequencing. Hemocytes that expressed wildtype levels of *A2bp1* were used as a control (WT hemocytes) while hemocytes expressing a single *A2bp1* RNAi construct were used to test for the effects of loss of *A2bp1* (*A2bp1* RNAi hemocytes). Groups of 60 adult female flies were uninfected, mock infected with PBS, or infected with live *Drosophila X Virus* or *S. aureus* (OD 5.0) for 3 hrs. Hemocytes were then isolated using immunoselection and cDNA was sequenced using 50-nucleotide, single-end reads on an Illumina platform (Figure 4-9A). It is important to note that *Drosophila X Virus* was included because 2 SNPs in *A2bp1* were identified in an unpublished *DXV* survival screen of the DGRP. However, *A2bp1*-silenced flies were not more susceptible to *DXV* infection nor did they show higher levels of *DXV* proteins after infection than control flies (Data not shown). Therefore, although the *DXV* samples were taken into account during the principal component analyses of the RNAseq experiment, the results of the *DXV* DE analysis will not be presented here.

Principal component analysis of all samples showed that the samples clustered according to genotype and treatment (Figure 4-9B). We began our analysis by examining gene expression changes in wild-type hemocytes after that occur after infection with *S. aureus* in wild-type hemocytes. Differential expression (DE) analysis was carried out to identify genes that were significantly (Benjamini and Hochberg (BH) $-\log_{10}$ adjusted *p*-value < 0.1) up or down regulated after *S. aureus* infection in wild-type hemocytes. To

obtain the list of highly differentially expressed genes, we compared the expression of mock infected and *S. aureus* infected hemocytes (Figure 4-10 and Tables 4.1 and 4.2).

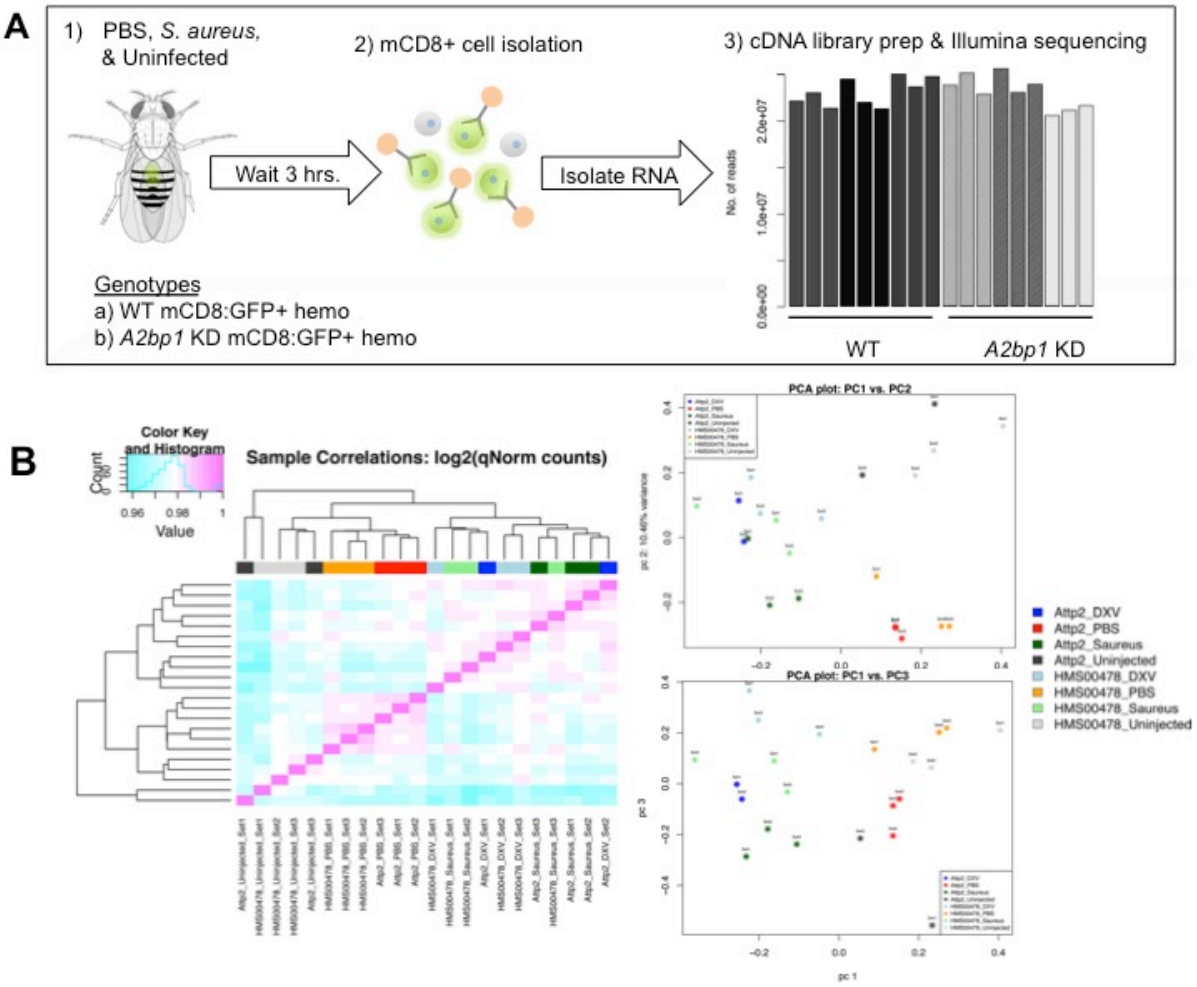

Figure 4-9: RNAseq and principal component analysis.

(A) Schematic of the workflow of the RNAseq experiment. (B) Left panel shows the correlation between gene expression profiles of RNAseq samples. Top right panel = PCA plot: PC1 vs PC2. Bottom right panel = PCA plot of PC1 vs PC3. See also Appendix B.

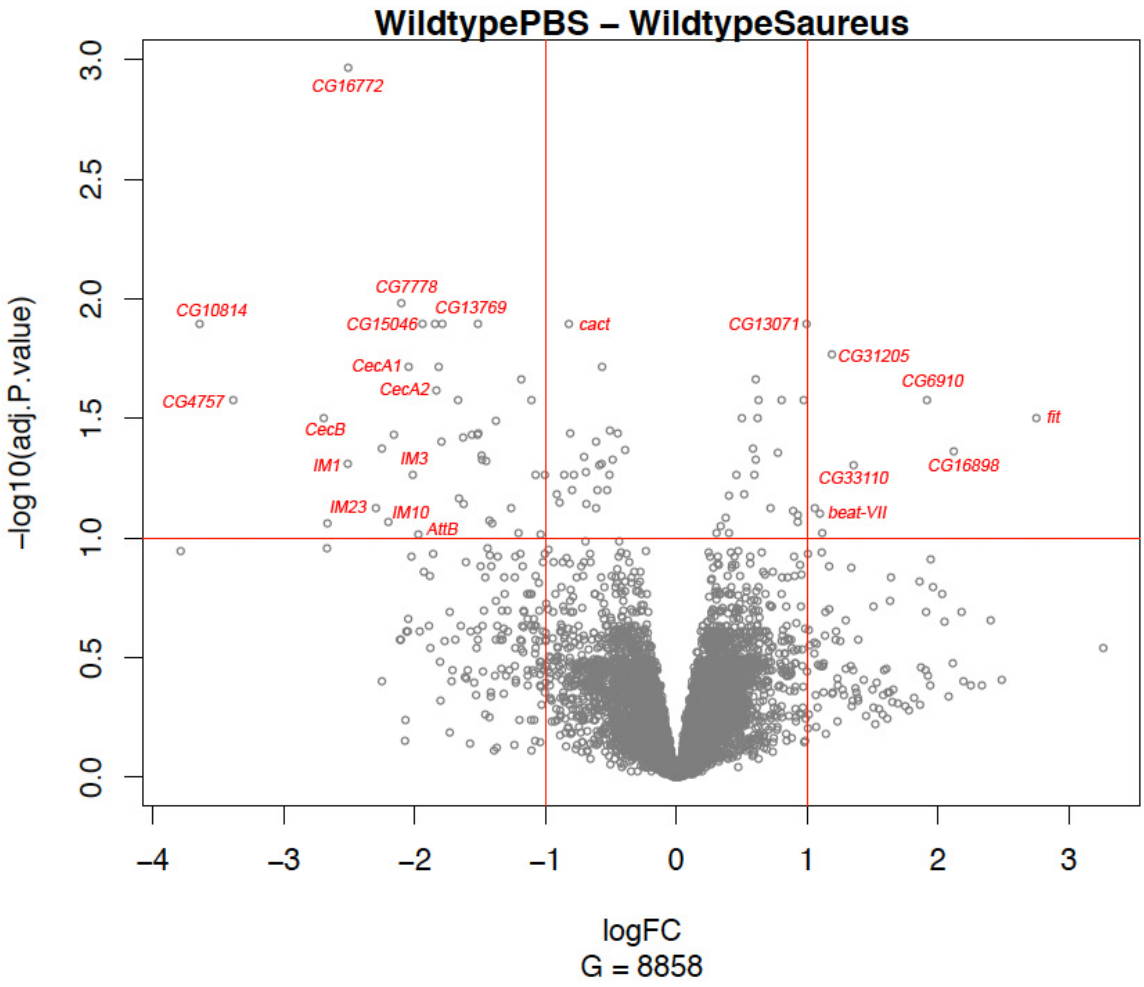

Figure 4-10: Genes differentially expressed in wild type hemocytes 3 hours after *S. aureus* infection.

(A) The differential expression between mock (PBS) infected and *S. aureus* infected wild-type hemocytes. The volcano plot depicts the magnitude of differential expression between WT and *A2bp1* hemocytes. Each dot represents a single gene (n= 8858) that is expressed in hemocytes. The horizontal line marks the threshold (Benjamini and Hochberg (BH) -$\log_{10}$ adjusted *P*-value < 0.1) for genes considered to be differentially expressed between samples. Genes with decreased expression in *S. aureus* infected hemoctyes have a positive log Fold Change (logFC) and genes with increased expression in *S. aureus* infected hemocytes have a negative logFC.

Table 4-1: Genes up-regulated after *S. aureus* infection in wild-type hemocytes.

Gene Symbol	logFC	FDR Adj. *p*-value	Flybase Gene Annotation
CG10814	-3.644	0.012	Gamma-butyrobetaine dioxygenase activity. Key enzyme in biosynthesis of L-Carnitine, a key molecule in long fatty acid chain metabolism.
CG4757	-3.383	0.026	Carboxylesterase, type B
CecB	-2.695	0.031	*Cecropin B*; Antimicrobial peptide; Humoral immune response
CG11459	-2.666	0.086	Cathepsin propeptide inhibitor domain (I29); Cysteine peptidase
IM1	-2.51	0.048	*Immune induced molecule 1*
CG16772	-2.5	0.001	Unknown
IM23	-2.29	0.074	*Immune induced molecule 2*3; Bomanin Family Protein
CG43202	-2.25	0.042	Unknown; Bomanin Family Protein
IMPPP	-2.20	0.085	*Immune induced molecule prepropeptide*, Toll mediated defense against
CG13965	-2.16	0.036	Unknown
CG7778	-2.10	0.0104	Unknown; Upregulated in larval hemocytes after Gram - infection;
CecA1	-2.04	0.019	*Cecropin A1*; Antimicrobial peptide; Humoral immune response
IM3	-2.01	0.054	*Immune induced molecule 3*; Bomanin Family Protein
AttB	-1.97	0.096	*Attacin-B*; Antimicrobial peptide; Humoral immune response
CG30002	-1.94	0.013	Peptidase S1, PA clan; serine-type endopeptidase activity
CG15046	-1.844	0.013	Peptidase S1, PA clan; serine-type endopeptidase activity
CecA2	-1.833	0.024	Cecropin A2; Antimicrobial peptide; Humoral immune response
Ser7	-1.815	0.019	Peptidase S1, PA clan; serine-type endopeptidase activity
Spn88Eb	-1.79	0.0396	Serpin family; serine-type endopeptidase inhibitor activity
CG42259	-1.787	0.0127	Hemolymph coagulation; Trypsin Inhibitor-like, cysteine rich domain
CG17167	-1.67	0.026	Transmembrane transport;calcium, potassium:sodium antiporter activity
CG15065	-1.661	0.069	Immune induced protein; Bomanin Family Protein
CG11842	-1.628	0.038	Peptidase S1, PA clan; serine-type endopeptidase activity
CG5791	-1.626	0.0723	Unknown; Bomanin Family Protein
IM4	-1.56	0.0369	*Immune induced molecule 4*
Unc-13-4B	-1.521	0.0369	Neurotransmitter secretion; synaptic vesicle priming
CG31769	-1.517	0.0127	Unknown; Found in hemolymph proteome
Vm34Ca	-1.511	0.0364	Structural constituent of vitelline membrane
Vm26Aa	-1.489	0.0449	Structural constituent of vitelline membrane
PGRP-SC2	-1.483	0.046	Negative regulation of the IMD pathway
CG13360	-1.452	0.048	Unknown; CHK kinase-like; Protein kinase-like domain
IM14	-1.427	0.085	Immune induced molecule 14
Hayan	-1.406	0.087	Regulation of melanization defense response; serine-type endopeptidase
AdoR	-1.377	0.032	Adenosine receptor; G-protein coupled receptor signaling pathway
Vm26Ac	-1.263	0.075	Structural constituent of vitelline membrane
dec-1	-1.205	0.096	Structural constituent of chorion
CG18067	-1.186	0.0218	Unknown; multicellular organism reproduction
NijA	-1.107	0.0264	*Ninjurin A*; Cell adhesion; Embryonic/larval lymph gland development
SPE	-1.075	0.054	*Spatzle-Processing Enzyme*
Ddc	-1.036	0.096	*Dopa decarboxylase*: cuticle pigmentation; response to wounding
Gadd45	-1.006	0.054	*Growth arrest and DNA damage-inducible protein GADD45*; JNK cascade

CG6967	-0.913	0.066	Nuclear-transcribed mRNA catabolic process, nonsense-mediated decay
capu	-0.891	0.0713	*capuccino;* Formin; actin binding; microtubule binding
Vml	-0.856	0.0543	Structural constituent of vitelline membrane
cact	-0.823	0.0126	*cactus;* Toll signaling cascade; Ankryin Repeat containing protein; Sequesters
CG8177	-0.811	0.0363	Inorganic anion exchanger activity
PGRP-LA	-0.798	0.0631	Peptidoglycan recognition protein LA; Positive regulation of IMD signaling
Bgb	-0.782	0.0545	Reluator of hemocyte proliferation; transcription coactivator activity
fend	-0.707	0.045	*Fork end*; motor neuron axon guidance
Ady43A	-0.690	0.053	Adenosine kinase activity; Catalysis of the reaction: ATP + adenosine = ADP
Rab19	-0.688	0.073	Vesicle-mediated transport; Rab protein signal transduction
CG12207	-0.615	0.0395	Unknown; LysM domain found in many proteins involved in bacterial cell
CG3165	-0.613	0.075	Nucleic acid binding; Ribonuclease H-like domain
sog	-0.605	0.0631	*Short gastrulation*; Growth factor activity; BMP and torso signaling
Fas3	-0.591	0.0497	*Fasciclin* 3; Immunoglobulin-like domain; cell-cell adhesion via plasma-
S2P	-0.572	0.049	*Site-2 protease*; sterol regulatory element binding protein cleavage;
tamo	-0.567	0.0191	*tamo;* Negative regulation of protein import into nucleus
spz	-0.529	0.063	*spatzle*; Toll receptor ligand
rogdi	-0.511	0.054	RAVE subunit 2/Rogdi; behavioral response to ethanol
loco	-0.506	0.035	G-protein signaling cascade; G-protein alpha-subunit binding
CG7115	-0.485	0.046	Cell adhesion; cation binding; protein serine/threonine phosphatase activity.
MICAL-like	-0.45	0.036	Calponin homology domain; actin binding
CG6923	-0.394	0.0428	Zinc ion binding; Zinc finger, RING-type

Table 4-1: A list of genes up-regulated after *S. aureus* infection in wild type hemocytes.

8859 *Drosophila melanogaster* genes were analyzed for differential expression (DE) between mock (PBS) infected and *S. aureus* infected wildtype hemocytes. False discovery rate (FDR) control was calculated using the Benjamini and Hochberg method. A cut-off of FDR adjusted p-value < 0.1 was used to obtain the list of 63 genes that are up-regulated in *S. aureus* infected hemocytes. logFC = log-Fold Change. Protein features were modified from Flybase.

Table 4-2: Genes down-regulated after *S. aureus* infection in wild-type hemocytes.

Gene Symbol	logFC	FDR Adj. *p*-value	Flybase Gene Annotation
ND-49	0.305	0.0955	*NADH dehydrogenase (ubiquinone) 49 kDa subunit*; mitochondrial electron
FoxK	0.337	0.0898	*Forkhead box K*; defense response to virus; positive regulation of gene
Jheh2	0.378	0.0821	*Juvenile hormone epoxide hydrolase 2*
alpha-	0.4	0.066	α-Mannosidase class I a; encapusulatin of a foreign target
CG6891	0.403	0.095	Actin-depolymerising factor homology domain
Samuel	0.46	0.054	SAM-motif ubiquitously expressed punctatedly localized protein; Regulation
P5cr	0.499	0.031	*Pyrroline 5-carboyxlate reductase*; Proline biosynthesis
bbg	0.519	0.0658	*Big bang*; PDZ domain; innate immune response in mucosa
foi	0.585	0.0424	*Fear-of-intimacy*; Zinc ion transmembrane transport
Sema-2a	0.595	0.0545	*Semaphorin-2A*; Sema domain and IgG-like domain; Secreted semaphorin;
rgn	0.605	0.0469	*Regeneration*; C-type lectin-like; carbohydrate binding
CG6084	0.606	0.0218	NADP-dependent oxidoreductase domain.
Obp99a	0.619	0.0315	*Odorant-binding protein 99a*
uzip	0.629	0.0264	*Unzipped*; axon guidance
CG15236	0.718	0.0751	Unknown
se	0.775	0.0443	*Sepia;* Glutathione S-transferase
Inos	0.803	0.0264	*Inos;* inositol-3-phosphate synthase activity
Nep1	0.89	0.077	*Neprilysin* 1; metalloendopeptidase
nord	0.926	0.0853	Fibronectin type III; Protein of unknown function DUF2369
Nlg2	0.927	0.0798	*Neuroligin 2*; Neuronal receptor activity
Gasp	0.972	0.0264	Structural constituent of peritrophic membrane; chitin binding
CG13071	0.994	0.0127	Unknown
CG31051	1.056	0.0749	Unknown
beat-VII	1.097	0.0786	IgG-like domain; heterophilic cell-cell adhesion via plasma membrane cell
CG34355	1.113	0.0955	DM13 domain; extracellular adhesion domain
CG31205	1.188	0.017	Peptidase S1, PA clan; serine-type endopeptidase activity
CG33110	1.354	0.0497	ELO family; very long-chain fatty acid synthesis
CG6910	1.916	0.0264	Inositol oxygenase
CG16898	2.12	0.044	CHK kinase-like; Choline kinase-like domain
fit	2.75	0.03	*female-specific independent of transformer*

Table 4-2: A list of genes down-regulated after S. aureus infection in wild type hemocytes.

8859 *Drosophila melanogaster* genes were analyzed for differential expression (DE) between mock (PBS) infected and *S. aureus* infected wildtype hemocytes. A FDR adjusted *p*-value < 0.1 was used to obtain the list of genes that are down in *S. aureus* infected hemocytes. logFC = log-Fold Change. Protein features were modified from Flybase.

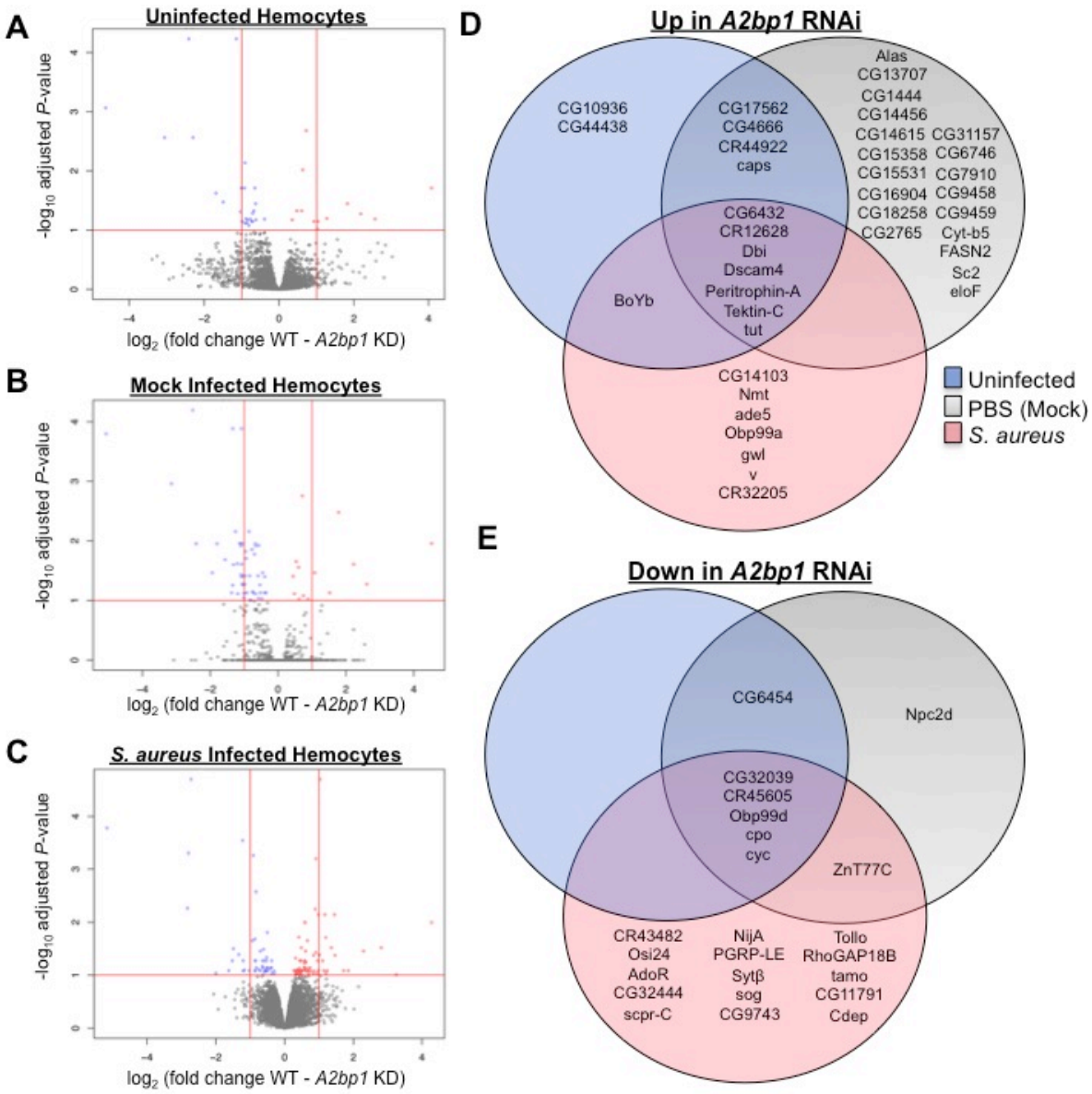

Figure 4-11: RNAseq and differential expression analysis reveal *A2bp1* targets in adult hemocytes.

(A) The differential expression (DE) between WT and *A2bp1* RNAi uninfected hemocytes. Genes with decreased expression in *A2bp1* RNAi hemoctyes are red and those with increased expression in *A2bp1* RNAi samples are blue. (B) DE between WT and *A2bp1* hemocytes 3 hours after PBS infection. (C) DE between WT and *A2bp1* hemocytes 3 hours after *S. aureus* infection. (D-E) Overlap of genes differentially expressed in each condition.

Sixty-three genes were up-regulated (FDR adusted *p*-value < 0.1) in adult hemocytes after *S. aureus* infection. These genes were significantly enriched for the Gene Ontology categories defense response [GO:0006952] (*p*-value 1.098e-10) and antibacterial humoral response [GO:0009607] (*p*-value 1.001e-07). Among the genes found enriched in these gene ontology categories were seven genes that encode proteins containing the Immune-induced protein Dim domain (*p*-value 3.46e-06): *IM1, IM3, IM14, IMPPP, IM23* and *IM4*. Additionally, we identified 4 antimicrobial peptide genes: *CecB, CecA1, AttB,* and *CecA2* and the Toll ligand *spatzle* as well as *Spatzle Processing Enzyme.*

Differential expression (DE) analysis was carried out to identify genes that were significantly (Benjamini and Hochberg (BH) $-\log_{10}$ adjusted *p*-value < 0.05) up or down regulated in *A2bp1* RNAi hemocytes compared to control hemocytes (Figure 4-11 and Table 4-3). If a gene's expression was down in *A2bp1* RNAi hemocytes, we concluded that splicing by A2bp1 is required to maintain the stability of mRNA expressed from that gene. Conversely, if A2bp1 mediates the decay or instability of a target mRNA, then we would expect to see higher levels in *A2bp1* RNAi hemocytes compared to controls.

Table 4-3. Putative *A2bp1* targets in *Drosophila* hemocytes.

Gene Symbol	logFC	FDR Adj. *P*-value	Flybase Gene Annotation
Up in *A2bp1*-RNAi hemocytes			
CG10936	-0.627	0.0357	SEA domain
caps	-0.648	0.0195	Leucine-rich repeat. Axon guidance.
CG4666	-0.676	0.0472	Belongs to the THEM6 family.
Dbi	-0.911	0.0073	ACB (acyl-CoA-binding) domain
BoYb	-0.921	0.0195	Helicase ATP-binding domain. ATP binding. DNA binding.
CG6432	-1.005	0.0195	AMP-binding enzyme C-terminal domain; AMP-dependent
CG17562	-1.027	0.0488	Fatty-acyl-CoA reductase (alcohol-forming) activity
CR12628	-1.142	5.88E-05	Microsomal glutathione S-transferase-like pseudogene.
CG44438	-1.489	0.0336	Unknown
CR44922	-1.684	0.0237	Non-protein-coding gene, Unknown Function
tut	-2.298	0.0027	RNA recognition motif domain
Dscam4	-2.409	5.88E-05	Fibronectin type III; Immunoglobulin I-set
Peritrophin-A	-3.054	0.00272	Chitin binding domain
Tektin-C	-4.628	8.59E-04	Microtubule binding
Down in *A2bp1*-RNAi hemocytes			
CR45605	4.082	0.0195	Non-protein-coding gene. Unknown Function.
Obp99d	1.827	0.0357	Pheromone/general odorant binding protein.
cpo	0.726	0.0021	RNA recognition motif domain.
cyc	0.631	0.0096	DNA binding. Nuclear translocator.
CG32039	0.603	0.0472	Unknown
CG6454	0.475	0.0472	C2 domain

Table 4-3: A list of putative *A2bp1* targets in *Drosophila* hemocytes.

8859 *Drosophila melanogaster* genes were analyzed for differential expression (DE) between WT and *A2pb1*-RNAi hemocytes. A FDR adjusted p-value < 0.05 was used to obtain the list of genes that are down in *S. aureus* infected hemocytes. logFC = log-Fold Change. Protein features were modified from Flybase.

We were primarily interested in how *A2bp1* regulates gene expression and splicing after *S. aureus* infection (Table 4-4). Twenty-two genes were down in *A2bp1* RNAi hemocytes after *S. aureus* infection, including all the genes whose basal expression was down in uninfected *A2bp1* RNAi hemocytes. In contrast, only 7 genes were down after PBS treatment. Five genes (*cpo, cyc, Obp99d, CR45605* and *CG32039*) were found significantly down in *A2bp1* RNAi hemocytes in all conditions, indicating that these five genes are the primary targets for A2bp1.

CR45605 was the most highly down-regulated transcript after *S. aureus* infection ($\log_2$ fold change of 4.281) and it encodes a computationally predicted long non-coding RNA (lncRNA) of unknown function. As an lncRNA, *CR45605* may play a role in blood cells by regulating the expression of genes important for phagocytosis of *S. aureus.* *Tollo/Toll-8*, *Peptidoglycan recognition protein LE* (*PGRP-LE*), and *tamo* are immune responsive genes that were among those that were significantly down in *A2bp1* mutant hemocytes after *S. aureus* infection (Akhouayri et al., 2011; Leulier and Lemaitre, 2008; Minakhina et al., 2003; Takehana et al., 2002). Tamo regulates the nuclear import of Dorsal in fat body cells after infection (Minakhina et al., 2003). *Tollo/Toll-8* and *PGRP-LE*, which both play a role during the humoral immune response to Gram-negative bacteria, are also up-regulated by A2bp1 in wildtype hemocytes after *S. aureus* infection. Thus, these genes may also function during the cellular response to Gram-positive bacteria.

Thirty genes were upregulated in *A2bp1* mutant hemocytes after PBS injection (Table 4-5). Within this group were six genes involved in the long-chain fatty acyl-CoA pathway: four members of the IPR002076 ELO family (*eloF, CG16904, CG9458,* and

CG9459), *CG1444,* and *Sc2* (Holm-Bonferroni corrected *p*-value = 6.76e-09). These results indicate that A2bp1 acts to down regulate fatty acid synthesis in hemocytes after wounding. Mammalian macrophages metabolize fatty acids to fulfill the energy needs necessary for activation and phagocytosis (Biswas and Mantovani, 2012). It is likely A2bp1 regulates hemocytes metabolic activity in response to wounding by diverting energy and resources from lipid metabolism. Furthermore, seven genes were up in *A2bp1* RNAi hemocyte samples in all conditions: *CG6432, CR12628, Diazepam-binding inhibitor* (*Dbi*)*, Peritrophin-A, tumorous testis* (*tut*), and *Dscam4.* Two of these genes, *CG6432* and *Dbi*, are also involved in synthesis and oxidation of fatty acids (Flybase annotations). Peritrophin-A is a constituent of the peritrophic membrane, tut is an RNA-binding protein, CR12628 is a non-protein-coding RNA, and Dscam4 is an immunoglobulin-like cell surface protein. This diverse group of genes are likely to be primary targets of *A2bp1* in wildtype adult blood cells, and *A2bp1* negatively regulates their expression.

Table 4-4. Genes differentially expressed in *A2bp1* RNAi hemocytes 3 hours after *S. aureus* injections.

Gene Symbol	logFC	FDR Adj. *p*-value	Flybase Gene Annotation
Up in *A2bp1*-RNAi hemocytes			
CG14103	-0.5024	0.0156	Unknown
Nmt	-0.525	0.0464	Glycylpeptide N-tetradecanoyltransferase activity
ade5	-0.613	0.0326	Phosphoribosylaminoimidazolesuccinocarboxamide synthase activity
BoYb	-0.704	0.0352	DEAD/DEAH box helicase domain; negative regulation of transposition,
Obp99a	-0.831	0.0027	Pheromone/general odorant binding protein
gwl	-0.862	0.0209	14-3-3 protein binding; protein binding; protein serine/threonine kinase
CR12628	-0.910	5.528E-04	Microsomal glutathione S-transferase-like pseudogene
CG6432	-0.934	0.02234	Acetate-CoA ligase activity
Dbi	-1.220	2.892E-04	ACB (acyl-CoA-binding) domain
v	-1.329	0.04089	Compound eye pigmentation, tryptophan 2,3-dioxygenase activity; heme
CR32205	-1.487	0.0317	Unknown
Dscam4	-2.714	2.00E-05	Immunoglobulin subtype 2,Fibronectin type III
tut	-2.791	5.005E-04	RNA recognition motif domain; Nucleotide-binding alpha-beta plait domain
Peritrophin-A	-2.819	5.451E-03	Chitin binding domain
Tektin-C	-5.155	1.688E-04	Microtubule binding
Down in *A2bp1*-RNAi hemocytes			
CR45605	4.281	0.0101	Unknown
CR43482	2.808	0.0305	Unknown
Osi24	2.292	0.0352	Unknown
Obp99d	1.463	0.0422	Pheromone/general odorant binding protein
ZnT77C	1.446	0.0072	Cation efflux protein transmembrane domain
AdoR	1.354	0.0305	G-protein coupled adenosine receptor activity
CG32444	1.196	0.0195	Aldose 1-epimerase activity; carbohydrate binding
scpr-C	1.168	0.0072	CAP domain; Cysteine-rich secretory protein, allergen V5/Tpx-1-related
cpo	1.028	2.00E-05	May play a role in the development of the peripheral nervous system by regulating the processing of nervous system-specific transcripts.
NijA	1.007	0.0415	Cell adhesion; tissue regeneration; embryonic/larval hemocyte
PGRP-LE	0.982	0.0072	Defense against gram negative bacteria
CG32039	0.912	6.364E-04	Unknown
Sytβ	0.909	0.03738	Calcium-dependent phospholipid binding; synaptic vesicle exocytosis;
sog	0.891	0.00567	von Willebrand factor, type C
CG9743	0.840	0.04223	Lipid metabolic process; oxidation-reduction process
Tollo	0.664	0.02239	Negatively regulates antimicrobial response in the Drosophila respiratory epithelium
RhoGAP18B	0.607	0.03523	GTPase activator activity
cyc	0.595	0.0101	Transcription factor involved in the generation of biological rhythms.
tamo	0.587	0.0101	Negatively regulates nuclear import of dl and controls the accumulation of dl
CG6454	0.485	0.0305	C2 domain, unknown function
CG11791	0.413	0.0192	Unknown
Cdep	0.320	0.0477	Rac protein signal transduction; regulation of Rho protein signal transduction

Table 4-4: List of genes DE in A2bp1 RNAi hemocytes 3 hours after S. aureus infection.

Table 4-5. Genes differentially expressed in *A2bp1* RNAi hemocytes 3 hours after PBS injections.

Gene Symbol	logFC	FDR Adj. *p*-value	Flybase Gene Annotation
Up in *A2bp1*-RNAi hemocytes			
Cyt-b5	-0.454	0.0345	Cytochrome b5, heme-binding site; Regulation of hemocyte differentiation.
Sc2	-0.568	0.0119	Lipid metabolism; oxidoreductase activity
CG1444	-0.588	0.0388	Short-chain dehydrogenase/reductase SDR
Alas	-0.661	0.0121	Chitin-based cuticle development; heme biosynthetic process; Aminotransferase,
caps	-0.667	0.0112	Leucine-rich repeat. Axon guidance.
CG6746	-0.694	0.0169	Protein-tyrosine phosphatase-like, PTPLA
CG2765	-0.770	0.0140	Myosin binding; Spot 14 family
CG15531	-0.838	0.0388	Fatty acid desaturase, type 1; Fatty acid desaturase, type 1, core
CG4666	-0.856	0.0070	Belongs to the THEM6 family.
FASN2	-0.942	0.0198	3-oxoacyl-[acyl-carrier-protein] synthase activity; fatty acid synthase activity; zinc ion binding; Oxidoreductase activity
CG6432	-0.959	0.0151	Acetate-CoA ligase activity
CG16904	-1.031	0.0388	ELO family; Fatty acid elongase activity
CG15358	-1.041	0.0112	C-type lectin; carbohydrate binding
CG13707	-1.075	0.0247	Unknown
CR12628	-1.079	0.0001	Microsomal glutathione S-transferase-like pseudogene
CG31157	-1.089	0.0371	Unknown
eloF	-1.098	0.0121	ELO family; Fatty acid elongase activity.
CG7910	-1.105	0.0111	Fatty acid amide hydrolase activity; carbon-nitrogen ligase activity, with glutamine
CG17562	-1.125	0.0110	Fatty-acyl-CoA reductase (alcohol-forming) activity.
CG14615	-1.199	0.0243	Transferase activity, Transferring acyl groups other than amino-acyl groups.
CG9458	-1.255	0.0070	Fatty acid elongase activity
CG9459	-1.314	0.0252	ELO family; fatty acid elongase activity
Dbi	-1.333	1.39E-04	ACB (acyl-CoA-binding) domain
CR44922	-1.559	0.0208	Unknown
tut	-1.801	0.0112	RNA recognition motif domain
CG14456	-1.939	0.0345	Unknown
CG18258	-2.413	0.0112	Carboxylic ester hydrolase activity
Dscam4	-2.517	6.38E-05	Immunoglobulin subtype 2; Immunoglobulin subtype; Fibronectin type III.
Peritrophin-A	-3.141	0.0011	Chitin binding domain
Tektin-C	-5.068	1.61E-04	Microtubule binding
Down in *A2bp1*-RNAi hemocytes			
CR45605	4.527	0.0111	Non-protein-coding gene, Unknown Function
Npc2d	2.231	0.0247	MD-2-related lipid-recognition domain; Immunoglobulin E-set
Obp99d	1.785	0.0034	Pheromone/general odorant binding protein
ZnT77C	1.082	0.0345	Zinc efflux transmembrane transporter activity
cpo	0.717	0.0018	RNA recognition motif.
CG32039	0.605	0.0279	Unknown
cyc	0.536	0.0223	Regulation of circadian rhythm. DNA binding. Nuclear translocator

Table 4-5: List of genes DE in A2bp1 RNAi hemocytes 3 hours after PBS infection.

E. RNAi of *Dscam4* rescues the impaired *S. aureus* phagocytosis of *A2bp1*-RNAi cells

Phagocytosis of microbes by immune surveillance cells is an immediate response that occurs within minutes of the initial infection. We hypothesized that *A2bp1* regulates the expression of cell surface receptors or co-receptors that may be important for *S. aureus* recognition and uptake. Thus, we focused our remaining studies on *Dscam4,* which encodes a plasma membrane transmembrane protein with Immunoglobulin-like (Ig-like) and Fibronectin type III (FN3) domains. There are four Dscam-like proteins in the *Drosophila* genome and the most extensively characterized of these is *Dscam1* (Armitage et al., 2012; Vogel et al., 2003). *Dscam1* has the potential to express over 18,000 alternative splice isoforms and is essential for axon guidance and the formation of neural connections in *Drosophila* (Graveley, 2005; Schmucker et al., 2000; Wojtowicz et al., 2004). An RNAi screen in the phagocytic S2 cell line identified 36 RNA-binding proteins that regulate alternative splicing of *Dscam1* but found that *A2bp1* had no effect on alternative splicing of *Dscam1* (Park et al., 2004), consistent with our finding that *A2bp1* RNAi in hemocytes does not alter *Dscam1* expression. *Dscam1* also has a role in the innate immune response, and is important for phagocytosis of *E. coli* in *Drosophila,* and for phagocytosis of *E. coli* and *S. aureus* in the *Anopheles gambiae* immune competent cell line, Sua5B (Dong et al., 2006; Watson et al., 2005). Additionally, RNAi depletion of *AgDscam* decreased survival and increased bacteria loads in mosquitos infected with *S. aureus* and *E. coli.* Based on our RNAseq data we hypothesized that *Dscam4,* and not *Dscam1*, is the Dscam family member regulated by *A2bp1* in

Drosophila hemocytes and, that this regulation is important for phagocytosis of *S. aureus*.

Dscam4 expresses 5 transcripts with variable 5' UTR sequences and alternatively spliced exons 21, 29, 31, and 32. Each isoform contains 9 extracellular Ig-like domains and 6 Fibronectin type III domains. The extracellular regions of the Dscam4 proteins are nearly identical (98 – 100% identity among isoforms). The main source of variability between Dscam4 isoforms is found in the cytoplasmic tail, after the transmembrane domain.

Dscam4 transcripts showed a 2.4 log-fold increase (FDR adjusted *p*-value 5.88E-05) in uninfected *A2bp1* RNAi hemocytes. Quantitative PCR analyses of larval and adult hemocytes confirmed that loss of *A2bp1* led to significantly increased levels of *Dscam4* mRNA (Figure 4-12A and B). If *Dscam4* overexpression contributes to the impaired cellular immune response of *A2bp1* mutant hemocytes, then decreasing the levels of *Dscam4* mRNA should rescue the phenotypes seen in *A2bp1* RNAi flies. Indeed, co-expression of RNAi constructs against *A2bp1* and *Dscam4* in the hemocytes, was sufficient to normalize *Dscam4* mRNA levels relative to control hemocytes (Figure 4-12C). There was a strong rescue of the phagocytosis phenotype in hmlΔ>*A2bp1* RNAi / *Dscam4* RNAi flies (Figure 4-12D). This data indicates that *Dscam4* expression is regulated by *A2bp1* and that reduction in *Dscam4* expression in *A2bp1* RNAi hemocytes facilitates phagocytosis of *S. aureus.* The increased level of *Dscam4* transcripts in *A2bp1* RNAi hemocytes may cause Dscam4 to be overly abundant on the surface of hemocytes. If Dscam4 is functioning as a negative regulator of phagocytosis, then an overabundance of the protein could lead to reduced uptake of *S. aureus. A2bp1* RNAi flies show

increased susceptibility and microbial growth after *S. aureus* infection and these phenotypes were partially rescued by co-expression of *Dscam4* RNAi (Figures 4-12E and F). During early time points, hmlΔ>*A2bp1* RNAi flies died significantly faster than hmlΔ>*A2bp1* RNAi/*Dscam4* RNAi flies, but both groups of flies succumbed to the infection at the same time (Figure 4-12E).

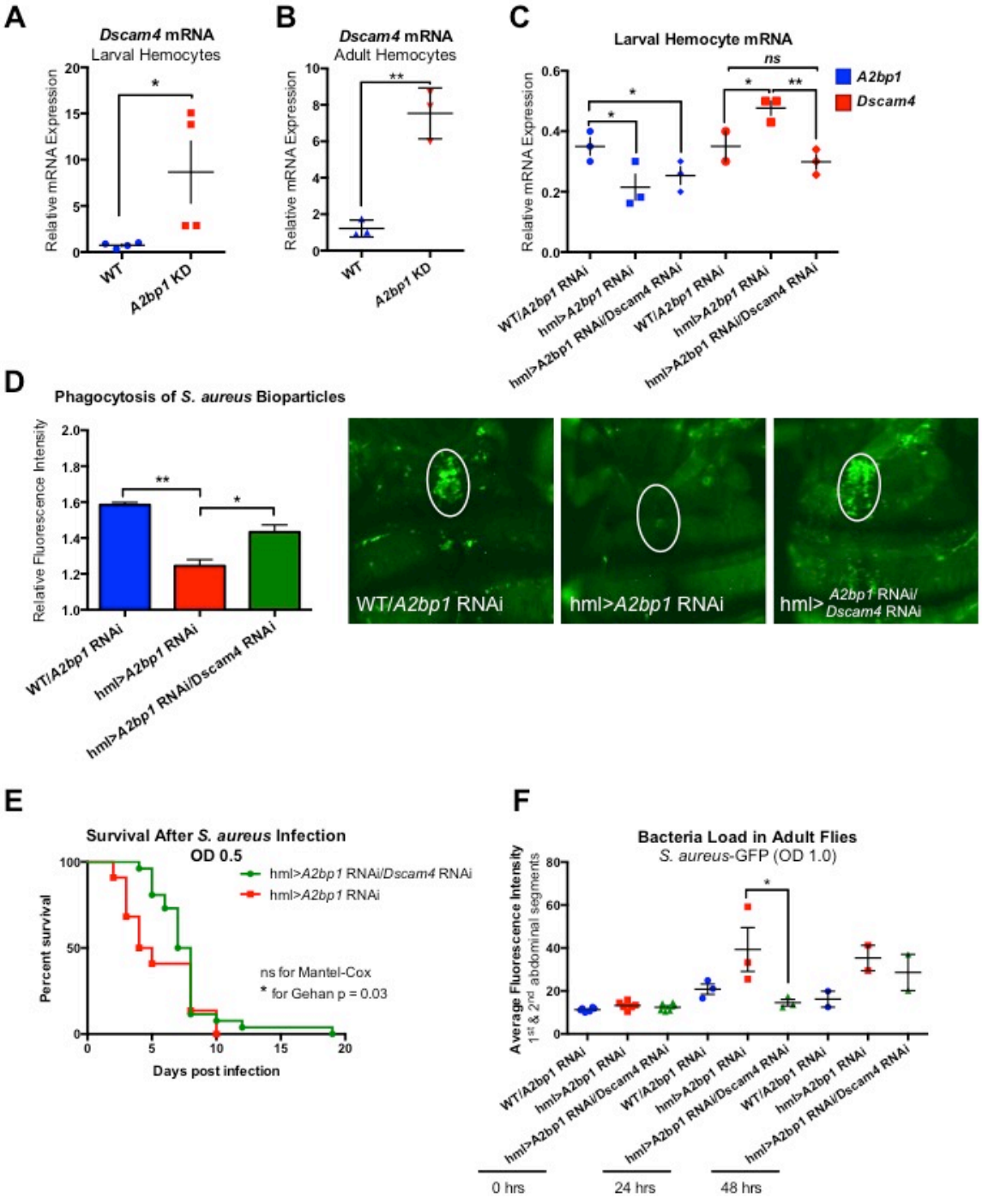

Figure 4-12: The immunoglobulin superfamily member *Dscam4* affects *S. aureus* phagocytosis.

Figure 4-12. The immunoglobulin superfamily member *Dscam4* affects *S. aureus* phagocytosis.

(A) Expression of *Dscam4* in larval hemocytes from WT/*A2bp1* RNAi and hmlΔ>*A2bp1* RNAi via qPCR. Hemocytes from 40 larvae were pooled for each experiment. The experiment was done 3 times. (B) *Dscam4* mRNA levels in adult hemocytes from WT/*A2bp1* RNAi and hmlΔ>*A2bp1* RNAi measured using qPCR. Relative expression was calculated using *rp49* as an endogenous control. All RNAseq samples were assessed: n=12 WT and n=12 *A2bp1* RNAi. (C) Expression of *A2bp1* (blue bars) and *Dscam4* (red bars) in larval hemocytes. Hemocytes from 10 larvae were pooled for each experiment. The experiment was performed in triplicate. (D) Quantification and representative images of phagocytosis of fluorescein-labeled *S. aureus* in WT/*A2bp1* RNAi, hmlΔ>*A2bp1* RNAi and hmlΔ>*Dscam4* RNAi */ A2bp1* RNAi flies. Six-to-eight flies were used in each experiment. (E) Representative survival curve of hmlΔ>*A2bp1* RNAi and hmlΔ>*Dscam4* RNAi */ A2bp1* RNAi flies after injection with *S. aureus* (OD 0.5) n = 28-30 flies. (F) Bacteria load in adult WT/*A2bp1* RNAi, hmlΔ>*A2bp1* RNAi and hmlΔ>*Dscam4* RNAi / *A2bp1* RNAi flies 0, 24 and 48 hours after *S. aureus* expressing GFP (OD 1.0) injections. For each fly, the fluorescence intensity of the first two segments of the dorsal side of the abdomen was measured. Approximately 16 flies per genotype per time point were used in each experiment. Error bars, ± SEM. * p-value<0.05, ** p-value<0.01, ns = not significantly different.

Similarly, blood cell specific co-expression of *A2bp1* RNAi and *Dscam4* RNAi rescued bacterial growth at 24 hours indicating that *Dscam4* is important to control bacteria growth at this time point. However, by 48 hours bacteria levels were similar in flies of both genotypes, revealing that *Dscam4* RNAi was not sufficient to control the growth of bacteria at the later stages of infection (Figure 4-12F). It appears that normalizing the levels of *Dscam4* in the *A2bp1* RNAi background is sufficient to protect the fly at early time points. Upon exposure to *S. aureus,* A2bp1 also regulates the expression of numerous other genes, including several immune-related transcripts known to play a role in the humoral immune response. Thus, other target genes of *A2bp1* may play additional protective roles at later stages during a pathogenic infection and this cannot be rescued by normalizing the expression of *Dscam4* alone.

III. Conclusion

We have presented a novel role for the RNA splicing factor, *A2bp1,* in the maintenance of phagocyte function and immunocompetence. RNA-binding proteins regulate immunological responses by altering the post-transcriptional processes of splicing, editing, decay and translation (reviewed in (Kafasla et al., 2014)). Either overexpression or silencing of *A2bp1* cause immune dysfunction, hinting that a delicate balance of *A2bp1* expression must be maintained to support hemocyte functions. Importantly, *A2bp1* is required to regulated post-transcriptional responses within *S. aureus* infected hemocytes.

Both mouse and human homologs of *A2bp1* negatively auto-regulate to maintain cellular homeostasis. This auto-regulation is achieved through an alternative splicing mechanism producing dominant negative isoforms that lack the RNA recognition motif

(RRM) (Damianov and Black, 2010). We observed a small reduction, approximately 20%, in *A2bp1* mRNA levels in hmlΔ>*A2bp1* RNAi hemocytes during our RNAseq experiment. This modest reduction may be due to *Drosophila* A2bp1 auto-regulating its own expression. It is possible that *A2bp1* RNAi results in the splicing of isoforms that are not important for the immune response in hemocytes, thus causing the differential expression of A2bp1 target genes.

Ultimately, *A2bp1* mutant flies are unable to control bacteria growth due to a reduced capacity to phagocytose *S. aureus.* This reduced phagocytosis is caused by an overabundance of *Dscam4* in mutant hemocytes. We propose that *Dscam4* may function as a negative regulator of *S. aureus* phagocytosis by serving as a receptor or co-receptor that inhibits the induction of signaling pathways that mediate uptake of the microbe.

In the fly, *A2bp1*-mediated splicing events are necessary to maintain hemocyte function in response to *S. aureus* infection. However, the immune-specific role for *Drosophila A2bp1* may also be relevant to human disease. *RBFOX1* is expressed in human peripheral blood cells and RRM of Drosophila A2bp1 and human RBFOX1, 2, and 3 are 94% identical (Bhalla et al., 2004; Kuroyanagi, 2009; Martin et al., 2007). Many immune genes and signaling pathways are conserved between humans and flies, raising the possibility that RBFOX proteins may also mediate the cellular immune response to *S. aureus* in human phagocytes.

IV. Materials and methods

Flies and fly husbandry: DGRP stocks were generated by Dr. Trudy Mackay's laboratory at North Carolina State University. The core 40 DGRP stocks (Ayroles et al., 2009) were provided by Dr. Jeff Leips' laboratory at the University of Maryland, Baltimore County. The UAS-*A2bp1-RE* flies were provided by Dr. L.S. Shashidhara from the Indian Institute of Science Education and Research in Pune, India (Usha and Shashidhara, 2010). The following stocks were obtained from the Bloomington Stock Center: w^{1118}, the blood cell-specific driver w^{1118}; *hmlΔGAL4,* y^1w^*; w^{1118}; P[*HemolectinΔGAL4*]2, P[UAS-2xEGFP]AH2, P[UAS-mCD8::GFP.L]LL5 (Stock #5137), y^1w^*; Mi[MIC]$A2bp1^{M101918}$ (#44669), w^{1118}; Df(3L)ED4457 (#9355), $y^1sc^*v^1$; P[TRiP.HMS00478]attP2 (#32476) and y^1v^1; P[TRiP.JF02600]attP2 (#27286). The *argus* mutant, and isogenic parental strain *cn bw,* were obtained from the EMS collection of Zuker lines (Koundakjian et al., 2004). UAS-*A2bp1-RE* and *A2bp1* RNAi lines were crossed to *hmlΔGAL4* flies or to w^{1118} as a control. Flies were reared at 25°C with 60% humidity under a 12 hour light-dark cycle and were fed a standard molasses/cornmeal/agar medium. Experiments were conducted at the same time each day.

***In vivo* phagocytosis:** To assay *S. aureus* and *E. coli* phagocytosis, approximately 6-8, 3-5 days old adults flies per genotype per experiment were injected with either fluorescein-conjugated *S. aureus* resuspended in PBS (Invitrogen: S2851, 1.6 mg/ml) or fluorescein-conjugated *E. coli* resuspended in PBS (Invitrogen: E2861, 1.6 mg/ml). Injections were performed using a Pneumatic PicoPump PV820 (World Precision Instruments). After 30 min, flies were injected again with Trypan Blue to quench extracellular fluorescence,

mounted ventral side down, and images of the dorsal vessel were taken using a Zeiss stereomicroscope (Discovery V8) with an AxioCam Hc camera. Fluorescence intensity of the area around the dorsal vessel was quantified using Axiovision 4.7 and the background fluorescence of an adjacent area was also quantified. The ratio of dorsal vessel fluorescence intensity was calculated as: $[\text{fluorescence}]_{\text{dorsal vessel area}} \div [\text{fluorescence}]_{\text{adjacent background area}}$. Two-tailed *t*-tests were used to calculate *p*-values.

To assay phagocytosis of beads, flies were injected with approximately 1.0μm Red Fluorescent Carboxylate Modified FluoSpheres diluted 1:20 in PBS. After 30 min, flies were injected with Trypan blue and then mounted and visualized as described above.

Genome-wide association analysis: To identify candidate SNPs that contribute to differences in *S. aureus* phagocytosis, we tested 30 lines of the DGRP using the *in vivo S. aureus* phagocytosis assay. We submitted the median fluorescence intensity phenotypes to the DGRP Freeze 1 Release 5.49 analysis pipeline (http://dgrp.gnets.ncsu.edu/) and GWA was performed as described in Mackay *et al.* 2012 (Mackay et al., 2012). SNPs were previously identified by whole-genome sequencing of the DGRP lines (Mackay et al., 2012) and SNP positions were annotated according to Flybase Release 5.49. We updated the position of the SNP in *A2bp1* to reflect the most current genome release, Flybase 6.07.

Larval phagocytosis assay: For each experiment, ten 3rd-instar, wandering larvae were washed in PBS, dried, and placed onto apple juice agar plates. Larvae were then injected with equal volumes of fluorescein-labeled *S. aureus* (resuspended to 1.6 mg/ml in sterile PBS with 5% green dye). After 30 minutes, larvae were bled directly onto polylysine coverslips by gently nicking the cuticle and bleeding hemolymph directly onto coverslips.

After 2 minutes, carcasses were removed and the cells were fixed with cold 4% Paraformaldehyde in PBS. The fluorescence of bioparticles not taken up by the cells was quenched by briefly washing fixed hemocytes with 5% Trypan Blue in PBS. The cells were then washed in PBS and the slides were mounted with Prolong® with DAPI. Hemocytes were imaged using a 63X oil immersion lens on a Ziess LSM700 Confocal Microscope and DIC was used to visualize the cell boundary. Approximately 10 cells per larvae were imaged and individual bioparticles were counted. Experiments were performed in triplicate.

Survival after *S. aureus* infection: Groups of 24-30 adult flies, 5-7 days old, were injected with equal quantities of logarithmic phase culture of *S. aureus* (final OD = 0.1 or 0.5). Flies injected with PBS served as a wounding control. Flies were kept at 25°C, transferred regularly to new food, and death was assessed every 24 hours. The experiments were repeated at least 2 more times. Log-rank tests were used to determine if survival curves were significantly different and p-values <0.05 were deemed significant.

Bacteria load assays: Thirty to 50 adult flies per genotype were injected with equal quantities of logarithmic phase culture of *S. aureus* (final OD = 0.5). At 0 and 24 hours post injection, 6-8 flies from each group were immediately washed in 70% EtOH, rinsed in PBS, and homogenized in Luria-Berani media containing 1% Trition X-100. Homogenates were serially diluted and plated on Luria-Bertani agar plates. Plates were stored at 37°C overnight and colony-forming units per fly were calculated. One-tailed t-tests were performed and p-values < 0.05 were determined to be statistically significant. Experiments were done at least 3 times.

For *S. aureus*-GFP bacteria load experiments, flies were infected with *S. aureus* expressing GFP (OD 1.0). Flies were then kept at 25°C and images were taken at 0, 24, and 48 hours post-injection using a Zeiss stereomicroscope (Discovery V8) with an AxioCam Hc camera. For each fly, the fluorescence intensity of the first two segments of the dorsal side of the abdomen was measured. At least 16 flies per genotype per time point were used in each experiment. Experiments were performed 3 times.

Fluorescence-Activated Cell Sorting (FACS) of Adult hemocytes

One hundred w^{1118} ; P[*HemolectinΔGAL4*]2, P[UAS-2xEGFP]AH2 flies (50 male and 50 female) were ground for 45 seconds in ice-cold PBS with 2mM EDTA. Cells were filtered through a 70μm cell sorter and centrifuged at low speed to pellet intact cells. Cells were then resuspended in ice-cold PBS/EDTA and filtered through a 40μm strainer. Cells were pelleted again and resuspended in PBS/EDTA. GFP-positive cells were sorted once using a FACSAria cell sorter with a 100μM nozzle and 20psi pressure. Cells were then re-sorted into the RLT buffer of the Qiagen RNEasy Mini Kit plus 3.75% Beta-mercaptoethanol. RNA was isolated according to the RNEasy Mini Kit protocol.

RNA isolation and Quantitative PCR

Adult RNA samples were obtained by homogenizing 6 flies (3 males and 3 females) in Trizol®. To obtain RNA from larval hemocytes and carcasses, 10 or 40 3rd-instar, wandering larvae were washed in PBS and bled by gently nicking the cuticle and bleeding hemolymph directly into PBS and Trizol® was immediately added. RNA was extracted from the adult and larval samples using Chloroform and Ethanol precipitation and loaded onto RNEasy Mini Columns. RNA was subsequently purified using the Qiagen RNEasy Mini Kit manufacturer's protocol. The RNA was digested with DNAse

(Thermo Fisher DNAse I, RNAse-free, EN05021) and cDNA was synthesized using reverse transcription (Thermo Fisher RevertAid First Strand cDNA Synthesis Kit, K1621). Quantitative real-time PCR was carried out using iQ SYBR Green Supermix (Bio-Rad) on an ABI 7300 following the manufacturers protocols.

Gene	Primer Sequence
A2bp1-RL, -RH, -RE, -RF, -RJ, -RI, -RK, and -RM	Forward: 5'GTCTCCAACATACCGTTCCG3'
	Reverse: 5'CATCGTTGCTGTTAGCGAATG3'
A2bp1-RL and *-RH*	Forward: 5'TTCACAAGAGCACGTCGATC3'
	Reverse: 5'GTGGGCGTTTATAGAGTGGG3'
Hemolectin	Forward: 5'GAGGACTAACAGCTTGGCAG3'
	Reverse: 5'CGGCATGAGACGTCTTTATC3'
Dscam4	Forward 5'GGCATTTCTGGCTCTGATTTG3'
	Reverse: 5'CGATTTATGGGCAGCGTTTG3'
RP49	Forward: 5'GCAAGCCCAAGGGTATCGA3'
	Reverse: 5' TAACCGATGTTGGGCATCAG3'

RNA-sequencing

Control flies (w^{1118}; P[UAS-mCD8::GFP.L]LL5, hmlΔGAL4; attP2) expressed the mCD8:GFP fusion protein on the surface of hemocytes and expressed normal levels of *A2bp1* in hemocytes. *A2bp1* RNAi flies were generated with the following genotype: w^{1118}; P[UAS-mCD8::GFP.L]LL5, hmlΔGAL4; P[HMS0048]attP2. *A2bp1* RNAi flies expressed both the mCD8:GFP fusion protein and the 21 bp short RNAi hairpin against *A2bp1* in hemocytes. Groups of 60 female flies were injected with equal volumes of *S. aureus* (OD 5.0) in sterile PBS or PBS (wounding control). Uninjected flies served as a control. Flies were incubated at 25°C for 3 hours and then homogenized in Cell Dissociation Solution (Sigma, C1544-100) in an RNAse/DNAse-free 1.5 ml tube with an RNAse-free mini-pestle for 45 sec. Supplemented Schneider's (0.1% BSA and 2mM

EDTA) was added to the homogenate and cells were filtered through a 70μm cell sorter and centrifuged at low speed to pellet intact cells. The cell pellet was resuspended in Supplemented Schneider's and incubated with Dynabeads Mouse CD8 (Lyt2) (Invitrogen, 11447D) for 30 minutes at 4°C. mCD8-positive cells were isolated using a magnetic stand and resuspended in RLT Buffer from the Qiagen RNeasy Mini Kit. Total RNA was then isolated according to the manufacturers protocols. Following cDNA library preparation, Illumina adaptors and indexes were added. Sequencing was carried out using an 50 base pair single-end reads on an Illumina HiSeq1000 system.

Differential Expression Analyses

I. Alignment and counting

Raw reads (51 nucleotides each) were trimmed 13 nucleotides from the 5-prime end using trimmomatic 3 (Bolger et al., 2014). Trimmed reads were aligned to the Flybase *D. melanogaster* genome (dmel-all-aligned-r6.03.fasta.gz) using Rsubread version 1.18.0 (Liao et al., 2013) allowing a total of 3 mismatches. The portion of total reads mapped for each sample is found in Supplemental Figure 3. Quantification was performed based on the Flybase annotation file (dmel-all-no-analysis-r6.03.gff.gz) using the featureCount (Liao et al., 2014) utility of Rsubread with default parameters to obtain a matrix of raw counts.

II. Normalization and differential expression

Genes with at least one count per million (cpm) in 3 (minimum number of biological replicates within a group) or more samples were kept and quantile normalized (Bolstad et al., 2003) for library size. Differential expression analysis

was performed using the voom-limma (Law et al., 2014) R package. In particular, the linear model *gene exprs = factor 1 + factor 2* was fit. Factor 1 has two levels: mutant and wild type; and factor 2 has 3 levels: PBS, *S. aureus,* and uninjected. Contrasts were used to assess comparisons of interest. All *p*-values were adjusted using the Benjamini and Hochberg (BH) method (Benjamini and Hochberg, 1996).

Chapter 5

Discussion

The initial line of defense in all animals is the innate immune response and phagocytosis of bacteria by specialized blood cells is a vital component of immunity. Several receptors have been implicated in microbial recognition and phagocytosis in Drosophila, but the study of phagocytosis is complicated by the redundancy of ligand binding specificities and overlapping functions of components of the phagocytic machinery.

To uncover novel genes in the cellular immune response, we conducted three genetic screens using the Drosophila Genetic Reference Panel (DGRP). The DGRP is a living library of polymorphisms across the entire genome and we conducted our screens as a complementary approach to previous RNAi and mutagenesis screens. The genetic polymorphisms within the DGRP are derived from naturally segregating genetic variation. Thus, the genetic variants within the DGRP may have more subtle effects than those found in mutagenized flies, where genomes have been altered by artificially induced mutations. RNA interference silences the expression of genes in target tissues and the phenotypes associated with RNAi reflect cell-autonomous loss-of-function effects. In contrast to RNAi, the variants within the DGRP may alter the function of genes in an assortment of ways; either by altering the expression of associated genes (by increasing or decreasing gene expression) or by changing the coding sequence of associated genes, resulting in the expression of proteins with altered amino acid

sequence. Thus, the DGRP allows us to identify effects of genes that may be undetected in traditional genetic screens.

We carried out two pilot screens using a subset of 30 DGRP lines, testing the ability of their hemocytes to recognize and phagocytose the Gram-positive bacteria *Staphylococcus aureus* or the Gram-negative bacteria *Escherichia coli.* We also screened 100 lines of the DGRP for the ability of their hemocytes to phagocytose and then clear infection of the Gram-positive bacteria *S. aureus* through phagosome maturation. We then performed genome-wide association analyses to identify potentially relevant single nucleotide polymorphisms (SNPs) that were significantly associated with the cellular immune response to *S. aureus* or *E. coli* within the DGRP.

To date, quantitative trait studies using the DGRP have tested morphological (sensory bristle number and cuticle pigmentation) (Dembeck et al., 2015), behavioral (olfactory response, locomotion, aggression, sleep, and alcohol sensitivity) (Arya et al., 2015; Harbison et al., 2013; Jordan et al., 2012; Morozova et al., 2015; Shorter et al., 2015; Swarup et al., 2013), and physiological (longevity, fitness, and resistance to oxidative stress, radiation, and starvation) (Durham et al., 2014; Ivanov et al., 2015; Vaisnav et al., 2014; Weber et al., 2012) traits in whole organisms.

Several studies have utilized the DGRP to identify polymorphisms that are associated with the immune response in whole flies. One study evaluated the genetic basis of variability in the immune response to viruses that naturally infect insects by measuring survival after viral infection (Magwire et al., 2012). This study identified several polymorphisms with large effects on resistance in the DGRP, including a non-synonymous SNP the gene *pastrel. Pastrel* is a gene that had not previously been

implicated in the immune response and it encodes a cytosolic protein that may be involved in constitutive protein secretion (Bard et al., 2006). Ubiquitous RNAi knock down of *pastrel* caused flies to become more susceptible to *Drosophila C Virus*, indicating that *pastrel* is important for antiviral immunity to *DCV*.

Another study used the DGRP to identify polymorphisms associated with the effects of nutrition on immunity to a natural bacterial pathogen (Unckless et al., 2015). The authors identified polymorphisms in the AMP gene *diptericin* and *defective proboscis extension response-6* (*dpr6*), a secreted Ig-like domain containing protein, that were associated with decreased resistance to the bacteria. We were intrigued that polymorphisms in *dpr6* were identified as important for the resistance to natural bacterial pathogens. We also identified two SNPs (smallest *p*-value = 2.18E-05) in *dpr6* that were important for the maturation of *S. aureus* containing phagosomes. In fact, *dpr6, dpr10, dpr1, dpr8,* and a protein that physically interacts with several members of the DPR-family (*Dpr-interacting protein α* (*DIP*-α)) were independently identified in our *S. aureus* phagosome maturation GWA. We suspect that the DPR-family, and DPR-interacting proteins, make up a group of immunoglobulin-like proteins that mediate the insect immune response to bacterial pathogens; a possibility that warrants further investigation.

More recently, a study to identify variants that affect survival to the Gram-negative entomopathogenic bacterium *Pseudomonas entomophilia* found that one extremely susceptible DGRP line, line 714, had a null mutation in *dredd* (Bou Sleiman et al., 2015). The loss of Dredd, the key caspase of the IMD pathway, caused line 714 to be highly susceptible to oral infection with multiple Gram-negative bacterial pathogens. It is possible that the null mutation in *dredd* may exist as rare heterozygous recessive allele in

the wild. However, due to the fact that DGRP lines are reared in a controlled laboratory environment, the homozygous null mutation in *dredd* was found in line 714. The genome-wide association analysis to identify SNPs that were associated survival to *P. entomophilia* found two SNPs in *Gyc76C,* a gene that encodes a membrane receptor that is highly expressed in Malpighian tubules and the midgut. Gcy76C is a receptor guanylate cyclase (rGC) that activates the IMD transcription factor Relish in response to stress, leading to the increased the expression of *Diptericin* (Overend et al., 2012).

Each of the studies mentioned above successfully identified known immune regulators as well as novel genes that play roles in the immune response in *Drosophila.* These studies demonstrate that the DGRP is a useful tool to identify genetic variants that affect *Drosophila's* immune response at the level of the whole organism. In contrast, we utilized the DGRP to conduct studies looking for genetic variants that affect a cellular process in one specific cell type, hemocytes. Our GWA yielded many new genes and pathways that give an overview into how hemocytes and phagocytosis are regulated in natural populations.

The *S. aureus* phagosome maturation screen identified over five hundred candidate genes whose predicted effects range from mediating hemocyte development to controlling pathogen recognition, uptake and degradation within hemocytes. The list of 528 *S. aureus* phagosome maturation associated genes was enriched for plasma membrane associated proteins and proteins with Ig-like motifs. We then tested the effects of 38 candidate genes: three associated with the most significant SNPs (p-value < 10^{-8}) identified in the *S. aureus* phagosome maturation screen, as well as a handful of genes that are predicted to localize to the plasma membrane or that encode proteins with Ig-like

domains. We tested genes associated with SNPs with p-value < 10^{-8} because these SNPs passed the significance threshold determined by Bonferroni multiple testing correction (p-value <0.05 after MTC). We also tested plasma membrane associated proteins because we were interested in characterizing proteins that can directly interact with *S. aureus* at the hemocyte cell surface. Finally, we tested proteins with IgG-like domains because many members of the Immunoglobulin superfamily play vital roles in microbial recognition, and this role is evolutionarily conserved. We used RNA interference to silence the expression of these select candidate genes in hemocytes, and we found three novel genes, *dpr10, fred,* and *CG42673,* whose loss-of-function in blood cells impaired the innate immune response to *S. aureus.*

Interestingly, our evidence suggests that *fred* and *dpr10* are not required for the initial uptake of *S. aureus,* but are instead required for the process of phagosome maturation to proceed after *S. aureus* is engulfed by the cell. This process seems to be specific to *S. aureus* as the loss of *fred* and *dpr10* affected the maturation of *S. aureus*-containing phagosomes but did not alter the maturation of *E. coli*-containing phagosomes. We hypothesize that *fred* or *dpr10* may be required for the proper intracellular trafficking or acidification of *S. aureus*-containing phagosomes, but the cellular mechanisms that mediate this specificity have yet to be determined. These findings support previous work that has shown cargo-specific phagosome maturation in mouse and human cell lines (Blander and Medzhitov, 2006; Zhu et al., 2015).

Future studies to examine the role of *fred* and *dpr10* should be conducted to verify the observed RNAi phenotypes. Immunofluorescence studies to assess *S. aureus* phagocytosis and phagosome maturation in purified larval or adult hemocytes expressing

RNAi against *fred* or *dpr10* will be necessary to confirm the phenotype observed in the adult *in vivo* phagocytosis and phagosome maturation assays. This study could also be carried out separately in male and females. We expect that hemocytes isolated from each sex will display phagocytosis phenotypes that mirror those observed in the adult *in vivo* phagocytosis assay. Analyzing phagocytosis in isolated hemocytes is a crucial experiment that allows us to visualize and quantify the effects of silencing candidate genes in single cells. Additionally, it may be useful to analyze the cellular immune phenotypes of null mutants or mutants with transposon insertions that affect the expression of *fred* or *dpr10.* These experiments will provide useful information about how gene disruptions that alter the expression of *fred* or *dpr10* affect *S. aureus* phagocytosis. We anticipate that lines in which the *fred* or *dpr10* loci have transposon insertions may display loss of function phenotypes that are similar or stronger than those observed when *fred* or *dpr10* are silenced in hemocytes via RNAi. Furthermore, rescue experiments in which wild type forms of *fred* and *dpr10* are expressed in mutant backgrounds should be carried out to confirm that wild type *fred* or *dpr10* are sufficient to restore the impaired immune response of mutant flies. Finally, to ascertain how Fred and Dpr10 mediate phagosome maturation it will be useful to generate tagged forms of each protein. Tagged Fred or Dpr10 can be used for both immunofluorescence and biochemical studies that will facilitate the functional characterization of each protein in hemocytes. Tagged forms of Fred and Dpr10 can be immunoprecipitated in order to identify their binding partners in *S. aureus* infected hemocytes. This study could yield valuable information about the dynamics of the phagosome and may assist in the

assembly of a model that supports a regulatory role for Fred and Dpr10 during maturation of *S. aureus*-containing phagosomes.

dpr6 and *dpr10* are the most closely related Dpr-family members identified in our *S. aureus* phagosome maturation screen. However, we did not test the effects of *dpr6* because an RNAi line for *dpr6* was not readily available when we conducted our secondary RNAi screen. We feel that it will be useful generate a line that expresses a short interfering RNA against *dpr6.* This line can be used to test for the immune effects of silencing *dpr6* in hemocytes. Owing to the high degree of similarity between *dpr6* and *dpr10,* it will be interesting to determine if these genes share a common role in the cellular immune response. Furthermore, another valuable study would be to examine a role for Dpr-family members in the cellular immune response to *S. aureus*. Multiple Dpr-family members were identified in our screen and the DGRP GWA to examining the immune response to natural pathogens (Unckless et al., 2015). We hypothesize that Dpr-family members may be important for microbial recognition in the fly. This project should begin with a small screen to assess the cellular immune effects of silencing the other Dpr-family members using RNAi in hemocytes. Additionally, because many Dpr-family members are predicted to be secreted from the cell, tagged Dpr proteins can be expressed and used in binding assays to determine if members of this family bind to bacteria *in vitro.* If this study reveals a clear physical interaction between the Dpr-family members and bacteria, it could signify that this family of genes is a class of antibody-like proteins that recognize microbes in insects.

CG42673 appears to play a role in the uptake of *S. aureus* by adult hemocytes. RNAi-mediated silencing of *CG42673* in hemocytes causes a decrease in the uptake of

S. aureus-fluorescein labeled bioparticles. CG42673 is not predicted to localize to the plasma membrane, but instead is predicted to be found in the cytosol. We hypothesize that the N-terminal phosphotyrosine-binding domain of CG42673 may be acting as a molecular scaffold to mediate the interaction between putative *S. aureus* receptors and downstream signaling molecules at the phagocytic cup. Experiments that are similar to those described above for *fred* and *dpr10* should be carried out to fully examine the cellular mechanisms by which CG42673 mediates the uptake of *S. aureus*. In particular, immunofluorescence studies to determine the localization of CG42673 in hemocytes, before and after *S. aureus* infection, should be carried out to confirm if CG42673 is acting at the phagocytic cup. Additionally, identification of CG42673 binding partners in hemocytes may reveal how it regulates *S. aureus* uptake. We suspect that CG42673 is acting downstream of either known or novel PRRs. CG42673 may interact with unidentified *S. aureus* receptors, and determining the cell-surface proteins CG42673 physically interacts with could potentially identify multiple new receptors for *S. aureus*. Additionally, it is possible that CG42673 recruits cytosolic signaling molecules to the phagocytic cup. By identifying the cytosolic binding partners of CG42673 we could potentially uncover signaling complexes that mediate uptake of bacteria. Finally, when *CG42673* is silenced in hemocytes, we observe a partial but significant decrease in *S. aureus* phagocytosis. Hemoctyes use multiple receptors to recognize *S. aureus*. If we find that CG42673 interacts with only a single *S. aureus* PRR, it would help explain why we only see a partial decrease in uptake of *S. aureus* when *CG42673* is silence in hemocytes.

The *E. coli* phagocytosis screen identified ten candidate genes that may be important for recognition or uptake by hemocytes. Based on the availability of transgenic

RNAi lines, we only tested the effects of 5 of these candidate genes using RNAi-mediated silencing in hemocytes: *Gγ30A, kuz, abd-A, CG16791,* and *CG5022*. We chose to silence the expression of candidate genes in hemocytes due to the fact that the trait we tested in our screen was specific to hemocytes located in the dorsal vessel of DGRP lines. We did not observe a difference in *E. coli* phagocytosis between control and knock down flies. However, it is possible that the genes we tested may be functioning in other tissues within the flies. Such a role would not be uncovered by targeting gene expression solely in hemocytes. In the future, it will be useful to test additional DGRP lines carrying the minor allele variant of SNPs associated with *E. coli* phagocytosis to see if these lines also exhibit altered *E. coli* phagocytosis phenotypes. These studies could confirm that the SNPs identified in our pilot screen are causal polymorphisms that must be present in order for DGRP lines to exhibit altered *E. coli* phagocytosis. This result would strengthen the results of our *E. coli* screen.

The small pilot GWA studies conducted by our lab used the same DRGP lines to test phagocytosis of *E. coli* and *S. aureus*. However, there was no overlap in the SNPs or genes associated with these phenotypes. Eighteen SNPs and 10 associated genes were identified in the *E. coli* GWA while only 3 SNPs and 3 associated genes were identified in the *S. aureus* phagocytosis GWA. It is feasible that the small sample size did not yield enough power to identify additional genes that affected phagocytosis of both types of bacteria. Eater is the only *Drosophila* receptor shown to affect phagocytosis of both *E. coli* and *S. aureus* in adult hemocytes (Kocks et al., 2005). It is possible that the DGRP lines we tested did not contain sufficient diversity at the *eater* locus for polymorphisms in this gene to be significantly associated with our phenotypes. Indeed, in order to be

included in the GWA analysis, the minor allele of each variant needed to be present in at least 4 of the lines exhibiting altered phenotypes.

Two genes identified in the *S. aureus* phagosome screen were also identified in the *E. coli* screen: *kuzbanian* and *CG42389.* Thus it is possible, that these two genes may be important for phagocytosis of both Gram-negative and Gram-positive bacteria. Two SNPs in *kuzbanian* (2L_13632604 and 2L_13632616 / smallest *p*-value = 8.84E-06) were associated with *E. coli* phagocytosis and another SNP in *kuz* (2L_13633995 / *p*-value = 8.23E-05) was associated with antagonistic *S. aureus* phagosome maturation within the DGRP. *kuzbanian* was identified as important for the phagocytosis of *E. coli* and the yeast *C. albicans* in an S2 cell RNAi screen (Stroschein-Stevenson et al., 2006). Kuzbanian cleaves Notch to generate a functional receptor during imaginal disc development (Sotillos et al., 1997). In larvae, Notch regulates the development of crystal cells but does not appear to affect the development of the phagocytic plasmatocyte lineage (Duvic et al., 2002). In contrast, during the adult stage, Notch signaling regulates the proliferation of dorsal vessel associated plasmatocytes. Thus, Notch affects the hemocyte niche in a distinct manner depending on the stage of development of the fly. The SNPs associated with *kuz* may alter Notch signaling and hemocyte development. Alternatively, kuz may be required for the activation of a transmembrane receptor that is important for phagocytosis of multiple types of bacterial cells. We carried out one experiment to test the effect of *kuz* on *E. coli* phagocytosis in adult hemocytes and saw no effect. However, due to the fact that multiple lines of evidence point to a potential role for *kuz* during phagocytosis, follow up experiments should be carried out to fully assess if there is a function for Kuzbanian during the cellular immune response.

Additionally, SNPs associated with the gene *CG42389* were identified in the *E. coli* phagocytosis GWA (2L_16630083 / *p*-value = 7.35E-06) and the antagonistic *S. aureus* phagosome maturation GWA (2L_16576978 / *p*-value = 4.21E-05). *CG42389* encodes a protein with Fibronectin III and Immunoglobulin-like domains. Very little is known about the function of CG42389 in *Drosophila* but a recent study using fluorescently tagged CG42389 reported that the protein localizes to the plasma membrane of ventral cells in the developing embryo (Lye et al., 2014). CG42389 was also reported to physically interact with Falafel, a regulatory subunit of *Drosophila* phosphoprotein phosphatase 4 (PP4) that has ben implicated in Rac1 signal transduction and phagocytosis of *C. albicans* (Lipinszki et al., 2015; Stroschein-Stevenson et al., 2006). Follow up work using RNAi to silence the expression of *CG42389* in hemocytes should be carried out to assess if there is an immune-specific role for this gene.

Two of our DGRP screens, the *S. aureus* phagosome maturation screen and the *S. aureus* phagocytosis screen, identified variants in *A2bp1* as important for the cellular immune response to *S. aureus*. *A2bp1* is a member of the highly conserved Fox-1 family of RNA-binding proteins. Members of the Fox-1 family bind to a consensus sequence, a (U)GCAUG element, in target RNAs and regulate tissue-specific alternative splicing (Auweter et al., 2006; Fukumura et al., 2007; Jin et al., 2003; Ponthier et al., 2006; Underwood et al., 2005). In Drosophila, A2bp1 i several developmental processes, including imaginal wing disc specification, female germline development, and nervous system development. Our study is the first report to describe a role for *A2bp1* in the innate immune response.

A2bp1 is expressed in larval and adult hemocytes. Studies using flies in which *A2bp1* was either silenced or overexpressed in blood cells indicate that the expression of *A2bp1* must be tightly controlled in these cells for proper function in these cells. Specifically, we observed strong defects in *S. aureus* phagocytosis both when *A2bp1* was silenced in hemocytes using RNAi or when *A2bp1-RE* was overexpressed in hemocytes. We suspect that as an RNA splicing factor, any alteration in the levels of A2bp1 exerts deleterious effects on target mRNAs, thus leading to defects in the immunocompetence of the hemocyte. Perturbation of *A2bp1* expression in hemocytes led to decreased phagocytosis of *S. aureus* and increased susceptibility to *S. aureus* infection. Using immunoselection and RNA sequencing, we analyzed the transcriptome of infected and uninfected hemocytes from wildtype flies and flies in which *A2bp1* was specifically silenced in hemocytes using RNA interference. We identified over 100 candidate genes that are differentially expressed in wild type hemocytes after *S. aureus* infection, many of which overlap with genes identified in previous hemocyte and whole animal transcriptome studies after bacterial infection (Boutros et al., 2002; De Gregorio et al., 2001; Irving et al., 2005).

Our transcriptome analysis generated a list of 20 genes that are likely the primary targets of A2bp1 in hemocytes. The cellular immune response is activated quickly in response to infection. As an RNA splicing factor, A2bp1 may control the expression of one or more proteins that mediate pathogen recognition and/or uptake. Indeed, one of the genes negatively regulated by A2bp1 in adult blood cells is the Immunoglobulin-superfamily member *Down syndrome adhesion molecule 4* (*Dscam4*). *Dscam4* levels are almost 4-fold higher in *A2bp1*-silenced hemocytes than control hemocytes. We stabilized

the level of *Dscam4* in *A2bp1*-RNAi hemocytes by simultaneously silencing of *A2bp1* and *Dscam4* in blood cells. By returning the levels of *Dscam4* to a normal range, we were able to rescue the fly's immune response to *S. aureus*. Thus, A2bp1 controls the level of Dscam4 in adult hemocytes and functional loss of A2bp1 leads to an over-expression of Dscam4, which in turn negatively regulates *S. aureus* phagocytosis.

The work we present demonstrates the utility of screening a wild population of flies to identify novel genes that play a role in the innate immune response to bacterial pathogens. Moreover, the genes identified in our study provide new insight into the functional mechanisms that underlie the cellular immune response to bacteria in *Drosophila melanogaster*. The *S. aureus* phagosome maturation GWAS found that many of the genes important for phagocytosis and neuronal development overlap. This overlap may be attributed to the existence of common cellular mechanisms that underlie phagocytosis and the outgrowth of neurons. We also observed that the cellular immune response is sexually dimorphic; with female DGRP lines showing higher levels of phagosome maturation and male lines showing lower levels of phagosome maturation. Studies of sexual dimorphism in the immune response of the fly have documented post-mating immunosuppression in both male and female flies (McKean and Nunney, 2001; Short and Lazzaro, 2010; Short et al., 2012; Vincent and Sharp, 2014). Humoral immune-related genes are upregulated in mated female flies compared to virgin flies (Lawniczak and Begun, 2004; McGraw et al., 2004). Studies reporting post-mating immune depression in female flies have noted that this trade-off is only seen after infection with certain bacterial pathogens (Short and Lazzaro, 2010; Short et al., 2012; Vincent and Sharp, 2014). Post mating immune depression was observed only after the flies were

infected Gram-negative *Providencia* species and not after infection with *Enterococcus faecilis* or *Pseudomonas aerurginosa*. Both *E. faecilis* and *P. aeruginosa* are recognized and phagocytosed by *Drosophila* hemocytes (Avet-Rochex et al., 2007; Nehme et al., 2011). It is unknown if the fly relies on the cellular immune response to defend against *Providencia* species infection, but *Providencia* are internalized by S2 cells indicating that these bacteria could be phagocytosed by hemocytes *in vivo* (Galac and Lazzaro, 2011). Thus, sexually dimorphic effects in the cellular immune response are observed after infection with some pathogens and not after infection with others. Survival outcomes or transcriptional responses after bacterial infections have been used to analyze the extent of sexual dimorphic immunity in *Drosophila*. Our *S. aureus* phagosome maturation GWA analyzed the genetic basis of sexual dimorphism in the cellular immune response to *S. aureus*. We report candidate SNPs and genes with sex specific effects on *S. aureus* phagosome maturation, and this list of genes may provide insight into the mechanisms governing sexually dimorphic responses to infection in *Drosophila.*

In conclusion, we have carried out multiple, interconnected projects in order to further our understanding of the molecules and signaling pathways that mediate the *Drosophila* cellular immune response to Gram-positive and Gram-negative bacteria. We have conducted genome-wide association screens to identify genetic variants that affect the cellular immune response in adult flies. We have also characterized roles for four new genes during phagocytosis of *S. aureus*. Finally, we successfully isolated adult hemocytes and analyzed gene expression patterns in immune challenged and unchallenged cells. We present our lists of GWAS candidate genes, the characterization of *fred, dpr10, CG42673* and *A2bp1,* and our hemocyte transcriptome analysis as a foundation for future work

studying the cellular immune response in *Drosophila melanogaster*. In both humans and insects, phagocytic blood cells act as the initial line of defense against bacterial infections. Characterizing the scope of the *Drosophila* cellular response to bacterial pathogens may provide insight into the functional mechanisms that underlie phagocytosis in humans.

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