LEENA-MAIJA VANHA-AHO

A Functional Study of the *Drosophila* Host Defense

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ACADEMIC DISSERTATION
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the Board of the BioMediTech of the University of Tampere,
for public discussion in the Small Auditorium of Building B,
School of Medicine of the University of Tampere,
Medisiinarinkatu 3, Tampere, on February 19th, 2015, at 12 o'clock.

UNIVERSITY OF TAMPERE

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Acta Universitatis Tamperensis 2022
Tampere University Press
Tampere 2015

UNIVERSITY OF TAMPERE

ACADEMIC DISSERTATION
University of Tampere, BioMediTech
Tampere Graduate Program in Biomedicine and Biotechnology
Finland

Supervised by
Professor Mika Rämet
University of Tampere
Finland

Reviewed by
Professor István Andó
Hungarian Academy of Sciences
Hungary
Docent Maria Vartiainen
University of Helsinki
Finland

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Cover design by
Mikko Reinikka

Distributor:
kirjamyynti@juvenes.fi
http://granum.uta.fi

Acta Universitatis Tamperensis 2022
ISBN 978-951-44-9710-0 (print)
ISSN-L 1455-1616
ISSN 1455-1616

Acta Electronica Universitatis Tamperensis 1511
ISBN 978-951-44-9711-7 (pdf)
ISSN 1456-954X
http://tampub.uta.fi

Suomen Yliopistopaino Oy – Juvenes Print
Tampere 2015

"Lights will guide you home
And ignite your bones"
-Coldplay

Contents

List of Original Communications

The thesis is based on the following original publications, which are referred to in the text by their Roman numerals. In addition, some unpublished results are presented.

I Ulvila J, **Vanha-aho L-M**, Kleino A, Vähä-Mäkilä M, Vuoksio M, Eskelinen S, Hultmark D, Kocks C, Hallman M, Parikka M, Rämet M. Cofilin regulator 14-3-3ζ is an evolutionarily conserved protein required for phagocytosis and microbial resistance. *J Leukoc Biol* 89: 649-659 (2011).[1]

II **Vanha-aho L-M**[2], Kleino A[2], Kaustio M, Ulvila J, Wilke B, Hultmark D, Valanne S, Rämet M. Functional characterization of the infection-inducible peptide Edin in *Drosophila melanogaster*. *PLoS One* 7(5): e37153 (2012).

III **Vanha-aho L-M**, Anderl I, Vesala L, Hultmark D, Valanne S, Rämet M. *Edin* expression in the fat body is required in the defense against parasitic wasps in *Drosophila melanogaster*. Submitted manuscript (2014).

[1] The original publication I was also partly used in the doctoral thesis of Johanna Ulvila (University of Oulu, 2008)

[2] Equal contribution

Abbreviations

Abi	Abelson interacting protein
Act5C	Actin 5C
AMP	Antimicrobial peptide
AttA	Attacin A
BHI	Brain-heart infusion
cDNA	complementary DNA
CecC	Cecropin C
cfu	Colony forming unit
cpa	Capping protein α
CyO	Curly of Oster
Da	Daughterless
DAP	*meso*-diaminopimelic acid
Dif	Dorsal-related immunity factor
Dpt	Diptericin
Dredd	Death-related Ced-3/Nedd2-like protein
Drs	Drosomycin
Dscam	Down syndrome cell adhesion molecule
dsRNA	double-stranded RNA
Edin	Elevated during infection
FADD	Fas-associated death domain
FBS	Fetal bovine serum
Gcm	Glial cells missing
GNBP	Gram-negative binding protein
He	Hemese
Hml	Hemolectin
hop	hopscotch
$hop^{Tum\text{-}l}$	$hopscotch^{Tumorous\text{-}lethal}$
Iap2	Inhibitor of apoptosis 2
IκB	Inhibitor of κB
IKK	IκB kinase

JAK	Janus tyrosine kinase
JNK	c-jun N-terminal kinase
LB	Luria-Bertani
LPS	Lipopolysaccharide
msnCherry	MSNF9mo-mCherry
NF-κB	Nuclear factor κB
NimC1	NimrodC1
PAMP	Pathogen-associated molecular pattern
PGN	Peptidoglycan
PGRP	Peptidoglycan recognition protein
PRR	Pattern-recognition receptor
PSC	Posterior signaling center
ROS	Reactive oxygen species
Rel^{E20}	*Relish*^{E20} null mutant
RNAi	RNA interference
siRNA	short interfering RNA
SR-CI	Scavenger receptor class C homolog, type I
STAT	Signal transducer and activator of transcription
TAB2	TAK-binding protein 2
TAK1	TGF-β-activated kinase
TEP	Thio-esther containing protein
TCT	Tracheal cytotoxin
TLR	Toll-like receptor
TNFR	Tumor necrosis factor receptor
TotM	Turandot M
UAS	Upstream activating sequence
Ush	U-shaped
qRT-PCR	quantitative real-time polymerase chain reaction

Tiivistelmä

Ihmisen immuunijärjestelmä muodostuu synnynnäisestä ja hankitusta immuniteetistä. Synnynnäinen immuniteetti on tärkeä osa meidän immuunivastettamme, sillä se toimii ensilinjan puolustusjärjestelmänä taudinaiheuttajia vastaan. Synnynnäisen immuniteetin mekanismit perustuvat genomiin koodattujen reseptorimolekyylien kykyyn tunnistaa taudinaiheuttajien pinnalla olevia yleisiä rakenteita. Taudinaiheuttajien tunnistus aktivoi nopeasti synnynnäisen immuniteetin mekanismeja, joihin kuuluvat muun muassa immuunisolujen harjoittama mikro-organismien fagosytoosi, signalointireittien aktivoitumien sekä tulehdusvälittäjäaineiden ja muiden immuunivasteeseen osallistuvien molekyylien tuottaminen ja erittäminen. Toisin kuin synnynnäinen immuniteetti hankittu immuniteetti, johon kuuluvat esimerkiksi vasta-aineiden tuotto ja immunologinen muisti, käynnistyy vasta myöhemmin kehityksen aikana ja on synnynnäistä immuniteettia hitaampi reagoimaan taudinaiheuttajiin. Koska synnynnäinen immuniteetti on erityisen tärkeä yksilön elinkaaren alkuvaiheessa sekä myös infektion aikaisessa vaiheessa, synnynnäisen immuniteetin mekanismien tutkiminen on hyvin perusteltua.

Banaanikärpänen, latinankieliseltä nimeltään *Drosophila melanogaster*, on osoittautunut erinomaiseksi mallieläimeksi synnynnäisen immuniteetin tutkimiseen, koska siltä puuttuu täysin hankittu immuniteetti, ja koska synnynnäisen immuniteetin mekanismit ovat hyvin säilyneet evoluutiossa. Tämän tutkimuksen tarkoituksena oli määrittää valikoitujen, aikaisemmin laajamittaisisten geneettisten seulontojen avulla tunnistettujen geenien toimintaa banaanikärpäsen immuunivasteessa. Erityisesti keskityttiin tutkimaan fagosytoosiin liittyvän geenin *14-3-3ζ*:n (14-3-3 zeeta) ja infektiossa indusoituvan geenin *edin*in (*e*levated *d*uring *in*fection) merkitystä banaanikärpäsen immuunivasteelle. Käyttämällä RNA-häirintään perustuvaa kudosspesifistä geeninhiljennystä pystyimme osoittamaan, että kofiliinin säätelijänä toimiva 14-3-3ζ on tärkeä tekijä bakteereiden fagosytoosissa, ja että sen toiminta on evoluutiossa hyvin säilynyt. Tutkimuksemme osoitti myös, että fagosytoosi on olennainen osa immuunipuolustusta bakteeri-infektion yhteydessä, koska *14-3-3ζ*:n hiljentäminen herkisti banaanikärpäset bakteeri-infektiolle. Osoitimme lisäksi, että sekä bakteeri-infektio että *Leptopilina boulardi* -pistiäisen aiheuttama loisinfektio sai

aikaan *edin*in ilmentymisen banaanikärpäsessä. Laajamittaisten *in vitro* ja *in vivo* -kokeiden perusteella pystyimme kuitenkin osoittamaan Edinillä olevan vain vähäinen tehtävä bakteeri-infektion yhteydessä. Sen sijaan tutkimuksemme perusteella Edin säätelee banaanikärpäsen verisoluja pistiäisinfektion yhteydessä.

Tämä tutkimus osoitti 14-3-3ζ:n ja Edinin olevan uusia banaanikärpäsen immuunivasteeseen osallistuvia tekijöitä, jotka toimivat erityisesti soluvälitteisen immuniteetin säätelijöinä. Tutkimustuloksemme tuovat lisätietoa synnynnäisen immuunivasteen toiminnasta sekä soluvälitteisen ja humoraalisen immuunivasteen vuorovaikutuksesta. Tästä tutkimuksesta saatu uusi tieto voi tulevaisuudessa auttaa ymmärtämään paremmin myös ihmisen synnynnäistä immuunivastetta johtuen synnynnäisen immuniteetin mekanismien samankaltaisuudesta banaanikärpäsen ja ihmisen välillä.

Abstract

The human immune system constitutes of the innate and the adaptive immunity. Innate immune responses are an important part of our immune defense as they provide the first line of defense against pathogens. Innate immunity is based on the ability of genome-encoded receptors to recognize common features on the surface of pathogens. This leads to rapid responses in the host, including the phagocytosis of microorganisms by immune cells, the activation of signaling cascades and the production and secretion of cytokines and other effector molecules. In contrast, the mechanisms of the adaptive immunity, which include, for example the production of antibodies and immunological memory, arise later on in development and are slower to react to an immune challenge. Because of the crucial importance of the innate immunity in the early stages of an individual's life cycle, and also in the early stages of an infection, a profound understanding of the regulators and mediators involved in the innate immune responses is needed.

The fruit fly, *Drosophila melanogaster*, is an excellent model organism for studying the mechanisms of innate immunity, because it lacks adaptive immune responses and because the mechanisms of innate immunity are evolutionarily conserved. This study focused on the functional characterization of selected *Drosophila* genes that had been previously identified as novel immunity-related genes in large-scale *in vitro* screens. Especially, the mechanisms of function of the phagocytosis-related gene *14-3-3ζ* (14-3-3 zeta) and the infection-inducible gene *edin* (*e*levated *d*uring *in*fection) were studied. Using tissue-specific RNA-interference (RNAi) mediated gene silencing we were able to show that the cofilin regulator 14-3-3ζ is an evolutionarily conserved protein required for the phagocytosis of bacteria both in *Drosophila* larvae and in adult flies. Our study also showed that phagocytosis is required for an efficient immune response against bacteria, because silencing *14-3-3ζ* with RNAi sensitized the flies to bacterial infections. Additionally, we showed that the expression of *edin* is upregulated in response to both a bacterial infection and wasp parasitism by *Leptopilina boulardi*, although after both *in vitro* and *in vivo* analyses Edin proved to have only a minor role in the host defense against bacteria. Instead, we were able to demonstrate that Edin is an important determinant in the defense against wasp parasitism, where it acts as a regulator of *Drosophila* blood cells.

The present study identified 14-3-3ζ and Edin as novel mediators of the *Drosophila* host defense, where they were found to take part especially in the cellular immune response. Our findings add more clarity to the mechanisms of innate immunity and provide more evidence of an active interaction between the humoral and cellular arms of the immune defense. In the future, the knowledge obtained from this study may serve as a novel starting point for human research due to the ancient origin of the mechanisms of innate immunity and their evolutionary conservation from fly to man.

1 Introduction

Vertebrates, invertebrates, plants and even unicellular organisms have developed efficient mechanisms to protect themselves from hostile agents that threaten their homeostasis. Common to all of these organisms is the ability of the host to recognize these harmful disease-causing agents and to carry out elaborate defense mechanisms in order to ensure the survival of the host. Somehow, our immune system is able to protect us from various pathogens, but at the same time leave our beneficial commensal microbes alone, highlighting the importance of the tight regulation of the immune responses. Although our immune responses are energy consuming and basically constantly active, we rarely even think about the immune defense until something goes wrong. A pathogen might overcome the first line of defense, i.e. the innate mechanisms encoded in our genome, and cause a disease. Or, in the case of autoimmune diseases, the immune system slips out of control and causes an immune reaction even in the absence of an infectious agent.

Although an understanding of the immune system and its control is of crucial importance, immunology as a science is relatively young, having its roots in the late 18th century, when Edward Jenner discovered the vaccination against smallpox. For a long time, the study of the mechanisms of innate immunity was overshadowed by the interest in the adaptive arm of the immune defense, including the production of antibodies and the immunological memory. The use of genetically tractable model organisms such as the fruit fly, *Drosophila melanogaster*, has demonstrated the importance and efficiency of the innate immunity. Like in humans, the innate immune responses in the *Drosophila* are mediated by both humoral and cellular factors that include the production of antimicrobial peptides and the phagocytosis of invading microbes by professional phagocytes. Because the mechanisms of innate immunity are of ancient origin, the signaling cascades and other components involved in the regulation of innate responses in humans have their counterparts in the fruit fly, and function in a similar way in both organisms. Therefore, the fruit fly provides an excellent platform for studying the underlying mechanisms of innate immune responses.

This present study used the fruit fly, *Drosophila melanogaster*, as a model organism to investigate both the humoral and cellular arms of innate immunity. The objective

was to determine the significance of selected target genes that had been previously identified in immunity-related large-scale screens in our laboratory. Because of the similarities between the human and fruit fly innate immune systems, the results obtained in this study may in the future provide a better understanding of the immune response in humans.

2 Review of the Literature

2.1 Innate immunity

In our every-day life, we are constantly exposed to a variety of disease-causing agents, or pathogens that challenge our health. To fight off these unwanted guests, we are armed with different defense mechanisms. The external and internal epithelial surfaces of our body act as physical barriers that protect us from invaders. Due to their mechanical, microbiological or chemical nature these barriers serve as hostile environments for the pathogens. If a pathogen manages to breach the initial safeguards, it is faced with the cells and molecules of our immune system. Most of the time, our innate immune mechanisms manage to locally prevent the onset of an infection, and no symptoms are observed. However, if the pathogen is successful in evading the defense mechanisms, an infection occurs. In the course of an infection, our immune system aims to limit the spreading of the infection from the initial site and to eventually overcome it. In the course of evolution the immune system, consisting of both the innate and adaptive immunity, has developed elaborate strategies, which insure the cellular integrity and survival of the host.

The first line of defense in all multicellular organisms is provided by the innate immunity, which appeared in evolution before the adaptive immune system (Hoffmann et al., 1999). Innate immunity is the only form of immunity in invertebrates, and its basic features have been highly conserved throughout evolution, as similar mechanisms are found in invertebrates, vertebrates and plants. Innate immunity is based on the ability of the host to distinguish self from harmful non-self. This feature is essential for the host to be able to carry out efficient and rapid immune responses. The discrimination of non-self relies on the ability of a limited number of genome-encoded pattern-recognition receptors to recognize and bind conserved molecular patterns found on the surface of pathogens. The pattern recognition receptors are expressed by immune effector cells, such as macrophages, granulocytes and antigen-presenting cells. Originally, the theory of pattern recognition and the presence of these pathogen-associated molecular patterns, or PAMPs, was described by Charles Janeway Jr. in 1989 (reviewed in Medzhitov, 2009). Examples of these conserved patterns are bacterial lipopolysaccharides (LPS),

peptidoglycans (PGN) and DNA, double-stranded RNA (dsRNA) from viruses and mannans from yeast. A common feature for all of the PAMPs is that they are invariant and essential structures produced by a certain class of pathogen, and are not produced by the host. These features enable a germ-line encoded recognition receptor to distinguish basically all infections caused by a certain class of microbes carrying a given molecular pattern.

To date, several families of pattern-recognition receptors with different ligand specificities are known. One of the most notable groups of pattern recognition receptors are the Toll-like receptors (TLRs) found in mammals. They are required for the recognition of a variety of PAMPs and express different ligand specificities. For example, TLR4, one of the human homologues of the fruit fly Toll, recognizes LPS (Medzhitov et al., 1997). Other families include mannose-binding lectin (MBL) which is specific for microbial carbohydrates, RIG-like receptors that bind viral RNA and NOD-proteins that are involved in the binding of PGN (reviewed in Janeway and Medzhitov, 2002). Receptor-ligand binding on the effector cells induces rapid defense mechanisms consisting of the activation of immune-related cells, the production of signaling and effector molecules, the activation of proteolytic cascades and the phagocytosis of invading microbes, all of which aim at constraining and clearing the infection.

In vertebrates, if the innate defense mechanisms are not successful in eliminating the infection, the adaptive immunity comes into play. Adaptive immunity first appeared in cartilaginous fish around 500 million years ago, and its hallmarks are immunological memory, the clonal expansion of specialized lymphocytes, namely T and B cells, and the production of antibodies (Hoffmann et al., 1999). Unlike the innate immunity, the adaptive immunity specifically recognizes antigens, but due to the need of the clonal expansion of lymphocytes, adaptive immune responses are comparably slow, taking from three to five days to take effect. Therefore in vertebrates, the innate immunity is also especially important in controlling the replication of pathogens during the first days of infection. In addition to its crucial role in the early phases of an infection, the innate immunity plays a key role in inducing adaptive immunity in vertebrates.

As is discussed above, innate immunity is essential in the regulation of several aspects of immunity. Therefore, it is of paramount importance that it is tightly controlled as misregulation of innate immune responses can lead to disease. The overactivation of innate responses can cause inflammatory reactions and lead to several autoimmune diseases, such as asthma or arthritis. Innate immunity is also especially important for neonates, whose adaptive immune system is not yet fully

developed. Although traditionally the adaptive immunity has been studied more, interest in the mechanisms of innate immunity has increased significantly due to several advances and important discoveries made in the field. Because of the evolutionary conservation of the genes involved in innate immunity and the lack of an adaptive immune system in invertebrates, the fruit fly, *Drosophila melanogaster*, has proven to be an excellent model organism in deciphering the underlying mechanisms of innate immunity.

2.2 *Drosophila* as a research model

The fruit fly, *Drosophila melanogaster*, (Greek for dark-bellied dew lover) has been used as a model organism in the field of biology for more than a century. *Drosophila* research started in the laboratory of Thomas Hunt Morgan in the early 20th century, when he used simple crosses to study the genetics of the *white* mutation. Based on his work on the fruit fly, Morgan was able to discover the role of chromosomes in heredity, nowadays a basic concept of genetics, for which he was awarded the Nobel Prize in physiology or medicine in 1933. Since his time, many advances made in the field of heredity, developmental biology and immunity have been made possible due to the work done with *Drosophila*. Such ground-breaking discoveries have fueled the use of the fruit fly in the scientific community and consequently, it has become one of the most commonly used model organisms in the world.

The practical explanations for the success of the fruit fly as a model organism are relatively obvious. The fruit fly is a small invertebrate (ca. 3 mm), which is easy to propagate in large scale without the need for large and elaborate facilities making setting up a fly laboratory cheap and easy. As an invertebrate, it also raises few ethical concerns compared to vertebrate animal models. The handling of flies is fast and cost-efficient, the life cycle of the fruit fly being only around 8-10 days at room temperature. Their short generation time and the high fecundity make fruit flies a feasible choice for large-scale experiments and screening. Because its natural habitat consists of rotting fruit, the fruit fly is not especially sensitive to infections and it does not require a complex or costly diet, but can be maintained on easy-to-make food in the laboratory, most of such feeds consisting of syrup, yeast, agar and cornmeal. In addition, the fruit fly is not sensitive to changes in environmental conditions, such as temperature, humidity or a light-dark cycle, and can be therefore

maintained even in a standard laboratory without the need to purchase expensive incubators.

Due to the long history of fruit fly research, *Drosophila* genetics is relatively well-known, and its understanding has recently been greatly aided by the release of the complete *Drosophila* genome sequence (Adams et al., 2000) and the publication of the genomic sequences of twelve related *Drosophila* species (Drosophila 12 Genomes Consortium et al., 2007). The discoveries made by Morgan and his colleagues in the early 20th century paved the way to the development of a vast array of genetic tools that are now used by scientists world-wide (reviewed for example in Rubin and Lewis, 2000). These tools, which are nowadays considered standard modifications, include balancer chromosomes carrying visible mutation markers that make it possible to maintain flies carrying lethal mutations as heterozygotes, greatly facilitating genetic manipulations. Traditional EMS mutagenesis screening and the generation of transgenic flies with the aid of P elements (Rubin and Spradling, 1982; Spradling and Rubin, 1982) have further expanded the selection of methods. Large stock collections of mutants, deficiency lines and a genome-wide collection of RNAi (RNA interference) fly lines (Dietzl et al., 2007) are now publicly available for everyone at low cost broadening the opportunities for *in vivo* research. The expression of transgenes or RNAi constructs can be tightly controlled both temporally and spatially with the UAS-GAL4 (Upstream activating sequence) bipartite system, originally adapted from yeast (Brand and Perrimon, 1993). More recently, the CRISPR system, initially identified in bacteria and archaea, has been adapted to the fruit fly, further increasing the possibilities of genetic manipulations (Gratz et al., 2014).

Despite apparent differences between humans and fruit flies, many biological processes and structures are shared between the two species making *Drosophila* a lucrative model organism for studying different biological phenomena. Furthermore, because of the compact structure of the *Drosophila* genome (~14,000 genes) and low redundancy in its genes' functions, the fruit fly offers a good option for vertebrate models and mammalian cell culture systems. The high evolutionary conservation of basic biological mechanisms is also applicable to immunity and disease-related genes (Table 1). Indeed, it has been estimated that 77% of human genes related to disease have a counterpart in the fruit fly (Reiter et al., 2001). Also, the major innate immune responses of the fruit fly, such as the phagocytosis of bacteria and immune signaling pathways controlling the production of antimicrobial peptides (AMPs), have been conserved in evolution from fly to man. As an insect, the fruit fly does not have an adaptive immunity, but it is solely dependent on the mechanisms of its innate

immunity leaving out the possibility that adaptive immune responses would compensate and interfere with the study of delicate phenotypes related to innate immunity. During the last couple of decades, the use of the fruit fly as an immunological model organism has steadily increased culminating in the Nobel Prize in physiology or medicine, which was awarded to Jules A. Hoffmann for his discoveries on Toll signaling in the activation of the innate immunity. The discovery of the Toll receptor in the fruit fly (Nüsslein-Volhard and Wieschaus, 1980) and its role in the innate immunity (Lemaitre et al., 1996; Rosetto et al., 1995) of the fly eventually led to the discovery of Toll-like receptors (TLRs) in mammals and their role in immunity (Medzhitov et al., 1997). This discovery is only one good example of how discoveries made in the fruit fly can benefit the study and understanding of the mechanisms of innate immunity on a broader scale.

Table 1. Innate immune defense in humans and fruit flies

	Human	Fruit fly
Innate immunity	+	+
Immune cells	Macrophages, neutrophils, dendritic cells, eosinophils, basophils, mast cells, natural killer cells	Macrophage-like plasmatocytes, crystal cells, lamellocytes
Phagocytosis	+	+
Complement system	+	Some components found
Antimicrobial peptides	+	+
Pattern recognition receptors	TLRs, PGRPs, NOD-like receptors, RIG-like receptors	PGRPs, GNBPs
Immune signaling pathways	TNFR pathway	Imd pathway, several components have counterparts in the TNFR pathway
	TLR pathway	Toll pathway, several components have counterparts in the TLR pathway
Toll receptors	10 Toll-like receptors	9 Toll receptors, not all immunity-related, no function in pattern recognition
Adaptive immunity	+	-

2.3 The *Drosophila* immune system

In its natural habitat, *Drosophila* lives on decaying fruit, where it is exposed to a large variety of microbes. The physical barriers protecting the fly against the entry of invading pathogens are the epithelia beneath the chitin exoskeleton of the fly, and the epithelia in the tracheae and the intestine, which are constantly challenged by microbes from the environment (reviewed in Kuraishi et al., 2013). The physical barriers are complemented with a robust innate immunity, but the fruit fly lacks the mechanisms of adaptive immunity. The immune system of *Drosophila* relies on both humoral and cellular defense mechanisms that include the activation of immune signaling pathways resulting in the systemic and local production of effector molecules, such as AMPs and reactive oxygen species (ROS), the activation of proteolytic cascades leading to coagulation and melanization, as well as the phagocytosis and encapsulation of foreign objects by the *Drosophila* blood cells, called hemocytes (Figure 1). The humoral and adaptive arms of the *Drosophila* innate immunity are not separate systems, but they overlap and interact with each other.

2.3.1 Humoral response

2.3.1.1 *Drosophila* antimicrobial peptides

One of the hallmarks of the humoral response in *Drosophila* is the rapid production of antimicrobial peptides, or AMPs, in response to an infection. AMPs have been well conserved in evolution and since the isolation of the first AMP, Cecropin, in the moth *Hyalophora cecropia* (Boman et al., 1972; Hultmark et al., 1980; Steiner et al., 1981), hundreds of AMPs have been reported to have a role in innate immunity in insects, humans, plants and other multicellular organisms. The early work done by Hans Boman and his colleagues set the stage for the interest in the humoral immunity in insects, and later especially in that of *Drosophila*. Due to the advances made in the identification of *Drosophila* AMPs and their regulation, humoral immune mechanisms were a hot topic in immunological research in *Drosophila* for a long time. Nowadays, the aspects of humoral immunity and the regulatory mechanisms behind the production of AMPs are rather well characterized.

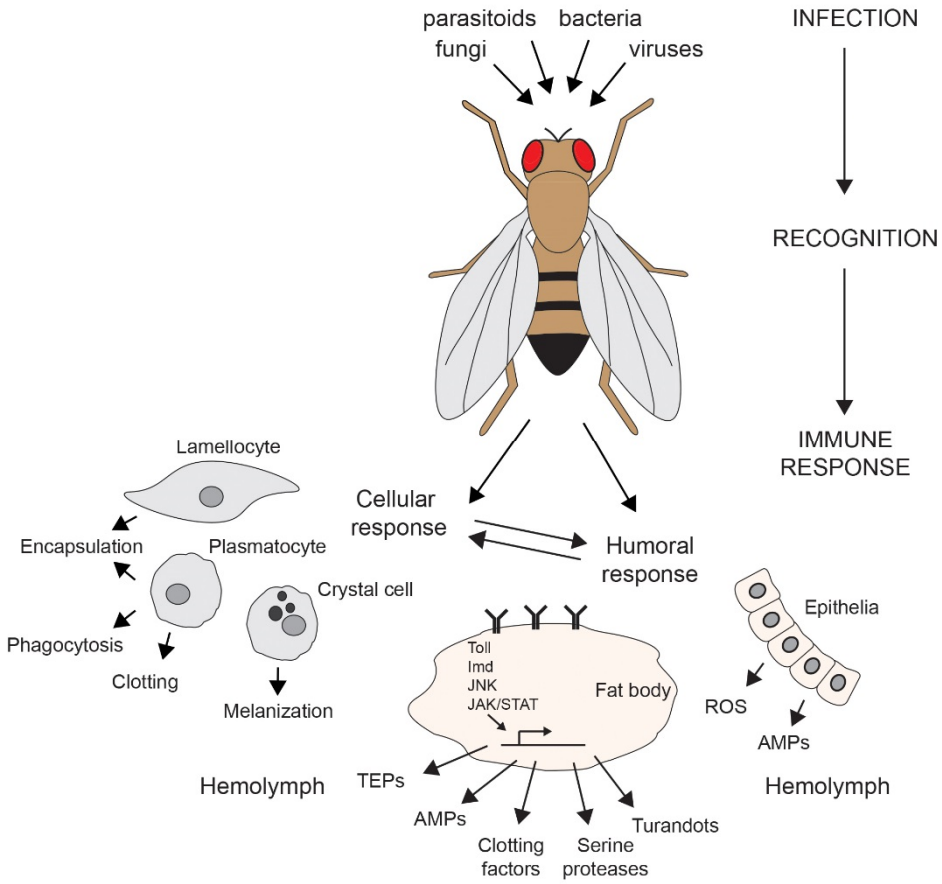

Figure 1. A simplified overview of the *Drosophila* immune response.

Drosophila can produce a large battery of AMPs that represent two functionally different classes; they are either antifungal or antibacterial (or both). The production of AMPs occurs either locally in the epithelial tissue or systematically in response to an infection. In general, AMPs are small peptides containing a signal sequence, which is cleaved off to produce a mature peptide before secretion. The promoters of *Drosophila AMP* genes contain regulatory elements similar to the mammalian NF-κB (nuclear factor κB) motifs that control acute-phase response genes (Engström et al., 1993; Kappler et al., 1993). To date, three members of the NF-κB transcription factor family that are required for the regulation of *AMP* gene expression have been identified in the fruit fly: Dorsal, Dif (Dorsal-related immunity factor) and Relish (Dushay et al., 1996; Ip et al., 1993; Reichhart et al., 1993). These transcription factors control the expression of seven classes of AMPs and their isoforms in the fruit fly: Attacin, Diptericin, Cecropin, Defensin, Drosocin, Metchnikowin, and

Drosomycin (Asling et al., 1995; Bulet et al., 1993; Dimarcq et al., 1994; Fehlbaum et al., 1994; Kylsten et al., 1990; Levashina et al., 1995; Reichhart et al., 1992; Tryselius et al., 1992; Wicker et al., 1990). More recently, a novel AMP was added to the list, as Listericin was reported to be active against the intracellular bacterium *Listeria monocytogenes* (Goto et al., 2010). Upon an infection, the production of AMPs is upregulated due to the nuclear translocation of the NF-κB transcription factors that activate immune signaling most notably via the Imd and Toll pathways, which are discussed in sections 2.3.1.3 and 2.3.1.4.

2.3.1.2 Systemic production of antimicrobial peptides and pattern recognition

The systemic release of *Drosophila* AMPs is controlled by an immune-responsive organ, the fat body, which is regarded to be the functional equivalent of the mammalian liver. The fat body is a relatively large organ that resides in the body cavity of the fly, and is surrounded by the circulating hemolymph, the insect blood. Because it is rich in nutrients, the hemolymph is a suitable growth environment for microbes. Therefore, it is paramount that the infection is recognized quickly. AMPs are produced rapidly in response to an infection and they are released into the surrounding hemolymph by the fat body. The presence of AMPs can be detected in the hemolymph within hours of an immune-challenge and their expression is again downregulated 12-24 hours after the infection to protect the flies from the detrimental effects of a prolonged immune response (Uttenweiler-Joseph et al., 1998).

The regulatory mechanisms behind the systemic production of AMPs by the fat body are already relatively well studied. The rapid production of AMPs in response to an infection is activated upon the recognition of harmful microbes by the *Drosophila* pattern-recognition receptors. Two families of pattern recognition receptors have been identified in *Drosophila*: the peptidoglycan recognitions receptors (PGRPs) and the Gram-negative binding proteins (GNBPs) (Kim et al., 2000; Werner et al., 2000), the latter being a historic misnomer, as they are mostly involved in the binding of fungal β glucans. The *Drosophila* genome contains 13 *PGRP* genes (only 4 in humans) coding for 19 proteins, and three *GNBP* genes (Kim et al., 2000; Werner et al., 2000; Werner et al., 2003). PGRPs were first identified in the silkworm, *Bombyx mori* (Ochiai and Ashida, 1999), and they can have either amidase or signaling activities, and are involved in the recognition of the bacterial cell wall component PGN. PGN is a polymer consisting of glycan strands of alternating molecules of β-1,4-linked *N*-acetylglucosamine and *N*-acetylmuramic acid cross-

linked together with short peptide bridges that have different compositions in different types of bacteria. The ability of *Drosophila* to discriminate between different types of bacteria and to mount a specific immune response is based on the ability of the PGRPs to recognize these different types of bacterial PGNs (Kaneko et al., 2004; Leulier et al., 2003). The stem peptides of most Gram-positive bacteria have a lysine-type PGN, whereas in most Gram-negative bacteria and in some Gram-positive bacteria the third lysine of the peptide bridge is replaced with *meso*-diaminopimelic acid (DAP-type PGN). PGRPs are classified either as short (PGRP-S) or long (PGRP-L) based on their transcript size (Werner et al., 2000), and they can be either transmembrane or secreted proteins. In addition to the PGRPs, a member of the GNBP family, GNBP1 is involved in the binding of lysine-type PGN together with two PGRPs, PGRP-SA and PGRP-SD (Bischoff et al., 2004; Gobert et al., 2003; Wang et al., 2008), whereas GNBP3 recognizes the long oligosaccharides of β-1,3-glucan (Gottar et al., 2006; Mishima et al., 2009).

Upon receptor-ligand binding, the signal is transduced via two major immune signaling pathways, the Toll and the Imd pathways, which control the expression of a differential set of AMPs and other immune responsive genes (Lemaitre et al., 1997). In addition, the JNK (c-Jun N-terminal kinase) and JAK/STAT (Janus tyrosine kinase/signal transducer and activator of transcription) pathways contribute to the expression of the target genes of the immune response (Boutros et al., 2002). Flies can mount a specific immune response based on the type of microbe that causes the infection: the Toll pathway is mainly activated by Lys-type PGN and β-glucans, which induce the production of antifungal and antibacterial AMPs, whereas the Imd-pathway is activated by DAP-type PGN resulting in the production of a set of antibacterial AMPs (Kaneko et al., 2004; Leulier et al., 2003). *toll* and *imd* mutants are especially sensitive to microbial infections (Lemaitre et al., 1995a; Lemaitre et al., 1996) indicating that both pathways are required for a normal immune response. In addition, flies that are deficient in both signaling pathways fail to induce the production of AMPs and succumb to a microbial infection (Lemaitre et al., 1996; Tzou et al., 2002). The regulation of the Imd and Toll signaling pathways will be discussed in the next two chapters.

2.3.1.3 The Imd pathway

One of the major pathways controlling the production of AMPs in the fruit fly is the Imd pathway (Figure 2), which is often compared to the mammalian TNFR (tumor necrosis factor receptor) pathway. The first component of the pathway was originally

characterized in 1995, when Lemaitre et al. identified *imd*, a mutant that was unable to induce the production of certain AMPs after a bacterial infection (Lemaitre et al., 1995a). The *imd* mutants, however, expressed the antifungal gene *Drosomycin* normally, implying the presence of another signaling pathway, which was later found to be the Toll pathway (Lemaitre et al., 1996). After the initial discovery of the *imd* mutant, the other components of the pathway started to unravel.

The Imd pathway is activated by DAP-type PGN (Kaneko et al., 2004; Leulier et al., 2003), which is recognized by the transmembrane receptor PGRP-LC and by the intracellular PGRP-LE. PGRP-LC is the major pattern recognition receptor for the Imd pathway and signals the presence of an infection (Choe et al., 2002; Gottar et al., 2002; Rämet et al., 2002b). PGRP-LC is alternatively spliced to produce three different isoforms: PGRP-LCx, PGRP-LCa and PGRP-LCy, which have similar transmembrane domains and intracellular signaling domains, but differ in their extracellular PGRP domains, which have different binding specificities (Werner et al., 2000; Werner et al., 2003). PGRP-LCx binds polymeric DAP-type PGN, whereas PGRP-LCa is not able to directly bind PGNs, but acts as a coreceptor for PGRP-LCx in the binding of the monomeric DAP-type PGN, called tracheal cytotoxin (TCT) (Chang et al., 2005; Chang et al., 2006; Mellroth et al., 2005). In addition to PGRP-LC, PGRP-LE acts as a receptor for DAP-type PGN in the Imd pathway, and it can be expressed both intra- and extracellularly. The cytosolic PGRP-LE is thought to be involved in the recognition of the PGNs of intracellular bacteria, such as *Listeria monocytogenes*, whereas the secreted form of PGRP-LE acts in collaboration with PGRP-LC in the recognition of extracellular PGNs (Kaneko et al., 2006; Neyen et al., 2012; Takehana et al., 2002; Takehana et al., 2004). Besides regulating Imd signaling, PGRP-LE is involved in the activation of the autophagy of intracellular bacteria (Yano et al., 2008).

The binding of bacterial DAP-type PGN to PGRP-LC leads to the dimerization of the receptor and the recruitment of the death-domain containing protein Imd, which shares homology with the mammalian RIP1 (receptor interacting protein) (Georgel et al., 2001). Imd interacts with the adaptor protein FADD (Fas-associated death domain) (Leulier et al., 2002; Naitza et al., 2002), which in turn binds and recruits the caspase-8 homolog Dredd (death-related Ced-3/Nedd2-like protein) to the signaling complex. Dredd is activated by ubiquitination by the ubiquitin E3 ligase Iap2 (Inhibitor of apoptosis 2) (Elrod-Erickson et al., 2000; Meinander et al., 2012). After activation, Dredd cleaves Imd and exposes a binding site for Iap2, which leads to the K63-polyubiquitination of Imd (Paquette et al., 2010). Most likely, the next

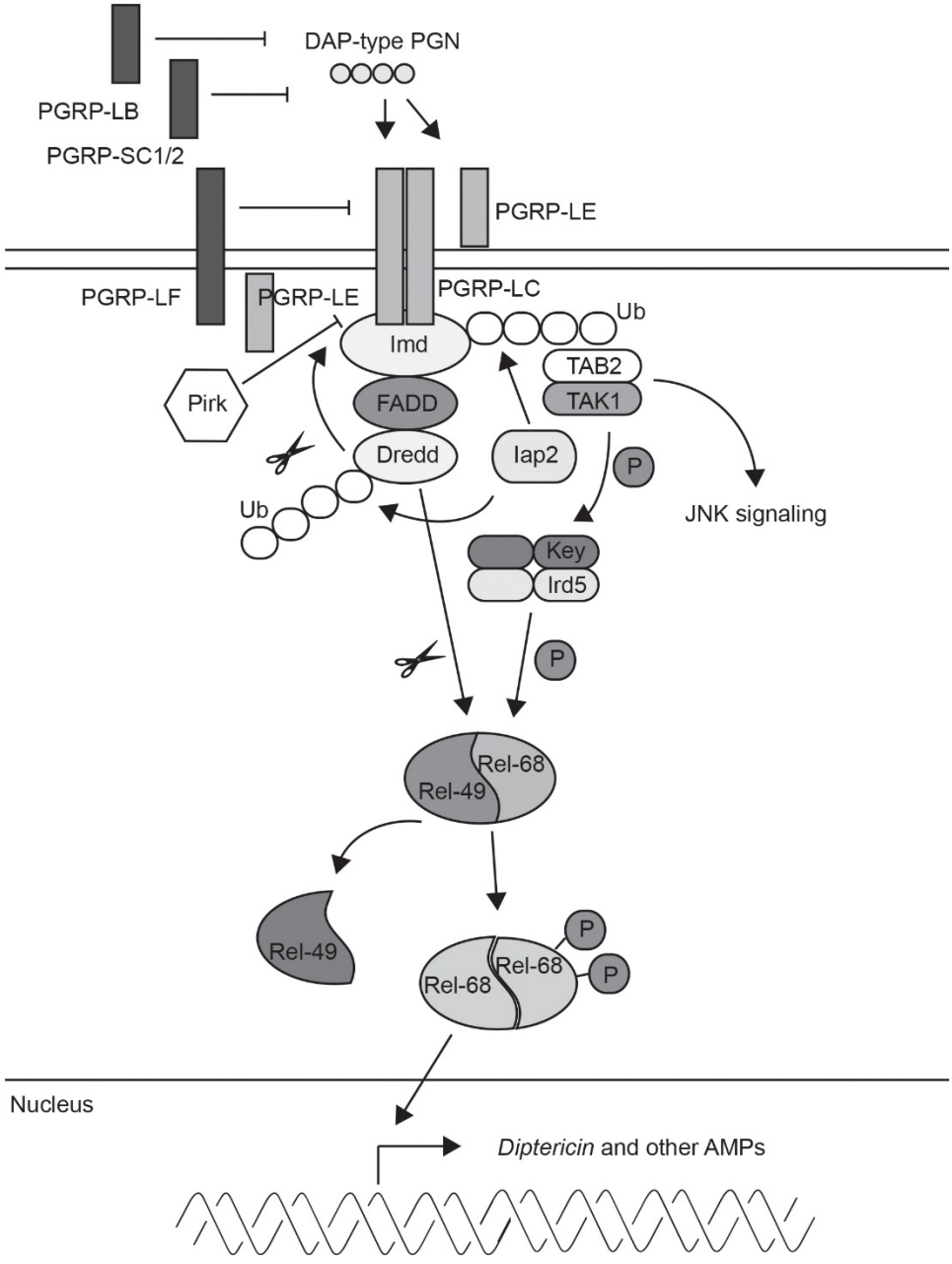

Figure 2. Schematic representation of the *Drosophila* Imd pathway. Some components have been omitted for clarity. Key= Kenny, Ubi= ubiquitination, P= phosphorylation

step in Imd signaling is the recruitment of the *Drosophila* homolog of the mammalian MAPK kinase kinase TAK1 (TGF-β-activated kinase) and its adaptor protein TAB2 (TAK-binding protein 2) (Kleino et al., 2005; Silverman et al., 2003; Vidal et al., 2001; Zhuang et al., 2006). The TAK1/TAB2 complex is involved in the phosphorylation of the *Drosophila* IKK (IκB kinase) complex, comprising of Kenny and Ird5 that are responsible of the phosphorylation of the NF-κB transcription factor Relish after it has been activated by Dredd through endoproteolytic cleavage (Kim et al., 2014; Silverman et al., 2000; Stöven et al., 2003). Once the C-terminal inhibitory ankyrin-repeat domain of Relish (Rel-49) has been cleaved off, the N-terminal domain of Relish (Rel-68) translocates to the nucleus, where it induces the expression of AMPs and other target genes (Stöven et al., 2000; Wiklund et al., 2009). Similar to the mammalian NF-κB pathways, the Imd pathway branches into the JNK pathway downstream of TAK1/TAB2 and is involved in the control of the early response and the induction of genes participating in cytoskeletal remodeling and stress responses (Boutros et al., 2002; Rämet et al., 2002a; Valanne et al., 2007).

Imd signaling is tightly regulated at several levels, which adds complexity to the signaling pathway. Various components of the pathway are subject to posttranslational, hormonal and negative regulation. A negative regulator of the Imd pathway, Pirk, is rapidly induced in response to an infection. This creates a negative feedback loop, which functions likely at the level of the PRGRP-LC/Imd/FADD signaling complex (Aggarwal et al., 2008; Kleino et al., 2008; Lhocine et al., 2008). The transcription factor Zfh1 has also been reported to function as a negative regulator of Imd signaling *in vitro*, but its role *in vivo* is less clear (Myllymäki and Rämet, 2013). Negative regulation of the Imd pathway occurs also at the level of PGRP-LC. PGRPs with an amidase activity, namely PGRP-LB, PGRP-SC1 and PGRP-SC2 downregulate the Imd pathway by digesting PGN into smaller fragments that have a decreased immunostimulatory activity and thereby reduce the activation of the pathway (Paredes et al., 2011; Zaidman-Remy et al., 2011). In addition to the amidase PGRPs, PGRP-LF can negatively regulate Imd signaling most likely by binding to PGRP-LC and by preventing its dimerization and hence, signal transduction (Basbous et al., 2011; Persson et al., 2007). In addition to the pathway being subject to negative regulation, Imd target genes are also transcriptionally controled in the fruit fly. In 2008, a *Drosophila* RNAi screen identified a previously unknown regulator of Imd signaling called Akirin, which acts downstream of Relish, but controls the expression of only a subset of Relish-dependent target genes (Bonnay et al., 2014; Goto et al., 2008). Recently, also ubiquitination and SUMOylation have been studied in the context of Imd signaling and they are now

known to be involved in the posttranslational modification of several Imd pathway components leading to either the activation or deactivation of the signaling cascade (Fukuyama et al., 2013; Kim et al., 2006; Meinander et al., 2012; Myllymäki et al., 2014; Thevenon et al., 2009; Tsuda et al., 2005).

2.3.1.4 The Toll pathway

The Toll pathway in *Drosophila* was first described in the context of the dorso-ventral patterning of the *Drosophila* embryo (Nüsslein-Volhard and Wieschaus, 1980; Nüsslein-Volhard et al., 1987), but nowadays it is known to also have several other roles in development. The first evidence for the involvement of the Toll pathway in immune signaling came in the 1990's, when Rosetto et al. showed that the Toll receptor has a role in activating the immune response in *Drosophila* S2 cells (Rosetto et al., 1995). The following year, Lemaitre et al. published their famous paper showing the involvement of the Toll pathway in controlling the production of the antifungal peptide Drosomycin (Lemaitre et al., 1996). The cover art of the paper, featuring a fly devoid of the Toll receptor dying of a fungal infection, nicely illustrated their main finding. The architecture of the *Drosophila* Toll pathway is the same in embryonic development and in innate immunity with the exception of the NF-κB transcription factor (Figure 3). The dorso-ventral patterning of the embryo is regulated by Dorsal, whereas the Dorsal-related immune factor (Dif) is involved in innate immunity (Ip et al., 1993). The discovery of the immunological role of the Toll pathway in *Drosophila* later led to the finding of the mammalian TLRs that are now known to operate in a key way in mammalian innate immunity (Medzhitov et al., 1997; Poltorak et al., 1998). Also, the other components of the *Drosophila* Toll pathway are evolutionary conserved, each of them having its mammalian ortholog. However, the opposite is not true, as the mammalian Toll pathway includes several components that are not found in the Toll pathway in the fly.

The *Drosophila* Toll pathway is activated by bacterial Lys-type PGN and fungal β-1,3-glucans. However, unlike the mammalian TLRs, the *Drosophila* Toll receptor does not function as a pattern recognition receptor. Instead, the PAMPs are recognized by secreted components upstream of the Toll receptor that activate proteolytic cascades leading to the cleavage and activation of the cytokine Spätzle, which is structurally and functionally related to the cysteine-knot protein nerve growth factor β found in vertebrates (Hepburn et al., 2014). The pattern recognition receptors implicated in the Toll pathway are GNBP3, which specifically binds β-1,3-glucans,

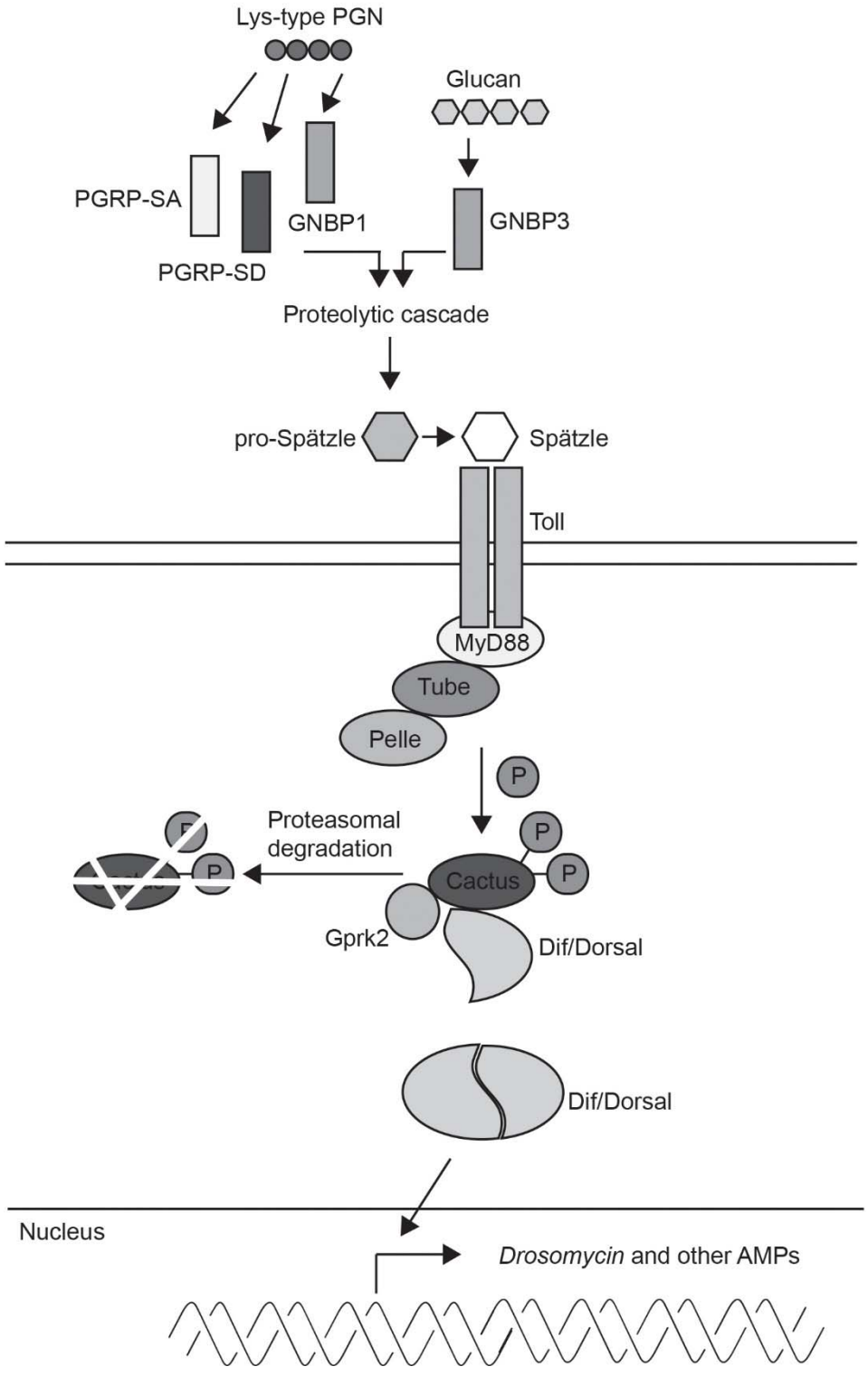

Figure 3. Schematic representation of the *Drosophila* Toll pathway.

and PGRP-SA, PGRP-SD and GNBP1 that recognize Lys-type PGN (Bischoff et al., 2004; Buchon et al., 2009c; Gobert et al., 2003; Michel et al., 2001). After the recognition and processing of Spätzle, the activated form of Spätzle functions as a ligand for the Toll receptor inducing a conformational change in the receptor and its dimerization (Gangloff et al., 2008). The activated Toll receptor then forms an intracellular signaling complex through its TIR (Toll/interleukin-1 receptor) domain with the adaptor proteins MyD88 and Tube and the kinase Pelle that interact with each other via their death domains (Horng and Medzhitov, 2001; Sun et al., 2002; Tauszig-Delamasure et al., 2002). The signaling complex is required for the phosphorylation of the inhibitory protein Cactus that is homologous to the mammalian IκBs (inhibitor of κB). In an unphosphorylated state, Cactus binds to the NF-κB transcription factors Dorsal or Dif and inhibits their nuclear translocation. Activation of the Toll pathway induces the phosphorylation of Cactus leading to its proteasomal degradation (Nicolas et al., 1998). For long, it has been under active investigation, whether Pelle actually is the kinase responsible for the phosphorylation of Cactus. This is the most likely explanation, as no other kinase has been so far implicated in Cactus phosphorylation (Valanne et al., 2010). This hypothesis is further supported by a recent paper by Daigneault et al. (Daigneault et al., 2013). The G-protein coupled receptor kinase Gprk2 was also found to interact with Cactus, but not to be required for the degradation of Cactus (Valanne et al., 2010).

After the phosphorylation of Cactus, the transcription factor Dorsal/Dif is released and translocates to the nucleus, where it interacts with target promoter sites through NF-κB binding motifs, the targets of which include *AMP* genes such as *Drosomycin* (Ip et al., 1993; Lemaitre et al., 1995b). In larvae, Dorsal and Dif have redundant roles in the immune response, whereas in adults Dif performs the task alone (Rutschmann et al., 2000).

2.3.1.5 Local production of AMPs

In addition to their systemic release in response to an infection, AMPs are produced also locally in epithelial tissues, where they are constitutively expressed (Tzou et al., 2000). Because the epithelia are constantly in contact with microbes, the constitutive expression of AMPs provides protection against opportunistic pathogens as well as against the normal bacterial flora of the fly. In contrast to the systemic production of AMPs by the fat body, the local production of AMPs in the epithelia is controlled by the Imd pathway, and not by the Toll pathway (Ferrandon et al., 1998; reviewed

in Kuraishi et al., 2013; Önfelt Tingvall et al., 2001; Ryu et al., 2006). The constitutive expression of *AMP* genes in the epithelia is not affected by an infection. In the case of a natural gut infection, the production of AMPs can be locally induced in the surface epithelia in a tissue-specific and Imd-pathway dependent manner (Buchon et al., 2009b). Even the expression of *Drosomycin*, one of the read-outs for Toll-pathway activity, is locally affected in the epithelia of *imd* mutants (Tzou et al., 2000). It has been proposed that, in addition to producing ROS by the dual oxidase pathway, the Imd-pathway controlled expression of AMPs in the *Drosophila* gut acts as a complementary antimicrobial defense system (Ha et al., 2005; Ryu et al., 2006). The Imd pathway together with the JAK/STAT pathway have also important roles in controlling gut tissue damage and renewal of the epithelium upon an oral infection (Buchon et al., 2009b; Ryu et al., 2006).

2.4 Cellular immunity

2.4.1 Drosophila hemocytes

The body cavity of the fruit fly is filled with circulating hemolymph, the equivalent of human blood. Contrary to mammals, *Drosophila* has an open circulatory system, where the *Drosophila* blood cells, called hemocytes, can circulate freely. Some hemocytes, however, remain sessile through the attachment to different tissues (Márkus et al., 2009; Zettervall et al., 2004). Because of the lack of adaptive immune responses, the fruit fly does not have a lymphoid lineage of blood cells, which in mammals is responsible for the production of antibodies and immunological memory. Instead, the fruit fly has three types of hemocytes, of which the predominant plasmatocytes resemble the mammalian macrophage lineage in their function, whereas the two others types, crystal cells and lamellocytes, do not have mammalian counterparts. The *Drosophila* hemocytes are involved in the phagocytosis of invading microbes and apoptotic corpses, the encapsulation of foreign objects as well as in the coagulation and melanization processes (Figure 1). Unlike mammalian blood cells, *Drosophila* hemocytes are not involved in the transport of oxygen; in flies this task is carried out by the tracheal system.

Plasmatocytes are the most abundant type of hemocytes in the fruit fly constituting up to 90-95% of all of the hemocytes, their total number depending on the developmental stage of the fly (Honti et al., 2014). Plasmatocytes are small and

round cells with a diameter of around 10 μm. They are the first hemocyte population to arise and are present at all developmental stages. Plasmatocytes act as professional macrophages in the fruit fly and are involved in the phagocytosis of small particles, such as invading microbes and apoptotic particles. Their function depends on cell-surface receptors that are capable of recognizing and inducing the phagocytosis of these particles (Ulvila et al., 2011b). In addition to their role as phagocytes, plasmatocytes are also involved in the humoral immune response and clotting by secreting AMPs and clotting factors (Dimarcq et al., 1997; Goto et al., 2001; Goto et al., 2003; reviewed in Theopold et al., 2014). Plasmatocytes show also remarkable plasticity by being able to differentiate into lamellocytes upon an immune stimulus (Honti et al., 2010; Stofanko et al., 2010).

Crystal cells represent a significantly smaller proportion of *Drosophila* hemocytes by constituting only around 5% of total hemocytes. Like plasmatocytes, crystal cells are small and round cells, yet nonphagocytic, and are instead involved in melanization. Crystal cells contain crystalline inclusions that are filled with prophenol oxidase, which in its active form catalyzes melanization reactions (Rizki and Rizki, 1959). Crystal cells are fragile and readily release their contents into the hemolymph upon activation by the JNK pathway (Bidla et al., 2007).

The third class of *Drosophila* hemocytes are lamellocytes, which are large and flat cells that are required for the encapsulation of objects that are too large to be phagocytozed by plasmatocytes. Lamellocytes are not found in the embryo or adult fly and are only rarely present in healthy larvae. Lamellocytes are formed in response to an immune signal such as a wasp infection or wounding (Lanot et al., 2001; Márkus et al., 2005; Rizki and Rizki, 1992). Together with plasmatocytes and crystal cells, lamellocytes form a multilayered capsule around the wasp egg and when successful, kill the parasite.

2.4.1.1 *Drosophila* hematopoiesis

Like in vertebrates, *Drosophila* hematopoiesis occurs in two temporarily and spatially different waves (Holz et al., 2003). The first phase of hematopoiesis takes place in the head mesoderm in the early embryo, where prohemocytes express the GATA transcription factor Serpent giving rise to around 700 embryonic plasmatocytes and 30 crystal cells (Rehorn et al., 1996; Tepass et al., 1994). Expression of U-shaped (Ush), an inhibitor of Serpent, as well as the transcription factors Glial cells missing (Gcm) and Gcm2 direct the prohemocytes to differentiate into plasmatocytes (Fossett et al., 2001; Lebestky et al., 2000). In contrast, prohemocytes that express

the transcription factor Lozenge, that suppresses Ush activity, differentiate into crystal cells (Ferjoux et al., 2007). The plasmatocytes migrate along well-studied routes and spread through the entire embryo phagocytosing apoptotic particles formed during development, whereas crystal cells remain clustered around the midgut and proventriculus, but their function in the embryo remains unknown (Franc et al., 1996; Lebestky et al., 2000; Siekhaus et al., 2010; Wood et al., 2006). The migration of plasmatocytes is also necessary for the proper development of the fruit fly nervous system (Evans et al., 2010).

Towards the end of embryogenesis, another hematopoietic organ, the lymph gland begins to form from the cardiogenic mesoderm providing a backdrop for the second hematopoietic wave that occurs in the larva (Lanot et al., 2001). The lymph gland is formed along the anterior part of the dorsal vessel, the *Drosophila* heart. In the early stages, the lymph gland consists of a single pair of lobes, called primary lobes that comprise of a limited number of plasmatocytes and crystal cells (Crozatier and Meister, 2007; Krzemien et al., 2010), whereas in the third instar larva secondary lobes develop posterior to the primary lobes. Larval hematopoiesis occurs in the primary lobes of the lymph glands, which consist of three separate zones (Jung et al., 2005). The posterior signaling center (PSC) comprises a small number of cells in the posterior end of the primary lobe expressing the ligand of Notch, Serrate, and the transcription factor Collier (Crozatier et al., 2004; Lebestky et al., 2003). The medullary zone contains precursor cells that are maintained in an undifferentiated state by both cell-autonomous and non-cell autonomous signals, whereas the differentiated hemocytes are located in the cortical zone alongside the outer edge of the lymph gland and arise from the progenitor cells (Jung et al., 2005). The cells of the PSC act in controlling hemocyte homeostasis and signal to the medullary zone to maintain the cells in their precursor state (Krzemien et al., 2007; Mandal et al., 2007). At least the activity of the JAK/STAT, wingless and Hedgehog signaling is required to keep the cells in the medullary zone in an undifferentiated state (Gao et al., 2009; Mandal et al., 2007; Minakhina et al., 2011; Sinenko et al., 2009). The differentiated hemocytes in the cortical zone are also involved in maintaining the precursor cells of the medullary zone in a pluripotent state by regulating at least the levels of adenosine (Mondal et al., 2011). In addition, nutritional signals, ROS and even olfactory signals have been associated with the regulation of hemocyte homeostasis (Owusu-Ansah and Banerjee, 2009; Shim et al., 2012; Shim et al., 2013). An immune challenge caused by a wasp infection activates the differentiation of prohemocytes in the lymph gland and the production of lamellocytes (Crozatier et al., 2004; Krzemien et al., 2010; Lanot et al., 2001; Sorrentino et al., 2002). Even in

the absence of an immune challenge, the prohemocytes in the lymph gland differentiate during metamorphosis and plasmatocytes and crystal cells are released into the circulation as the lymph gland disintegrates (Grigorian et al., 2011; Lanot et al., 2001). These hemocytes released during the early pupal stage persist through metamorphosis and participate in the immune responses of the adult (Charroux and Royet, 2009; Defaye et al., 2009; Holz et al., 2003). To date, no hematopoietic organ has been reported to exist in the adult.

In addition to the the lymph gland, the larval hemocytes reside in two other hemocytic compartments; in the sessile compartment and in circulation. The sessile cells form a distinct striped pattern under the integument of the larva consisting mostly of plasmatocytes and crystal cells (Márkus et al., 2009; Zettervall et al., 2004). The sessile cells represent a functional set of hemocytes that can be released into the circulation and that can rejoin the sessile compartment (Makhijani et al., 2011). The banded pattern is lost upon a wasp infection and the sessile cells are released into the circulation and differentiate into lamellocytes (Honti et al., 2010; Márkus et al., 2009; Stofanko et al., 2010; Zettervall et al., 2004). It has been proposed that during a wasp infection, it is actually the release of the sessile cells that is important in the early phases of the immune response against wasps (Honti et al., 2014), whereas the cells differentiating in the lymph gland might not play a role in parasitism. Most likely the circulatory hemocytes act as sentinels in the body cavity of the fruit fly signaling the presence of microbes or other harmful agents (Babcock et al., 2008). The majority of circulating cells constitutes of plasmatocytes, but also crystal cells are present in the circulation.

Blood cell homeostasis must be tightly controlled, as perturbations can cause significant defects in the fly. For example, certain mutations can cause the formation of melanotic tumors that resemble the capsule formed around a wasp egg. The formation of these melanotic masses in the fly resembles mammalian leukemias and is associated with an increase in hemocyte levels (Sorrentino et al., 2004). Especially, proper signaling via the Toll and JAK/STAT pathways is crucial as $Toll^{10b}$, $Cactus^{A2}$ and $Hop^{Tum\text{-}l}$ mutants are associated with the formation of melanotic masses (Luo et al., 1995; Roth et al., 1991; Zettervall et al., 2004).

2.4.2 Phagocytosis

Phagocytosis is one of the major cellular immune reactions carried out by *Drosophila* plasmatocytes. Phagocytosis constitutes an evolutionarily conserved innate immune

defense mechanism that has its origins in the uptake of nutrients by single-cell organisms (Hoffmann et al., 1999). Phagocytosis was first described in the late 19th century by the Russian immunologist Elie Metchnikoff, who observed that phagocytes, which he named macrophages, were able to ingest microbes. Macrophages are key components of the innate responses in all organisms, since they do not require prior contact with microbes. Phagocytosis is initiated by the recognition of microbes by cell-surface receptor molecules. This leads to downstream signaling events, remodeling of the actin cytoskeleton and internalization of the microbe by membrane restructuring. The phagosome then fuses with lysosomes to form a phagolysosome that causes the acidification of the environment and the destruction of the particle (Stuart and Ezekowitz, 2008; Ulvila et al., 2011b).

The fruit fly's professional macrophages, the plasmatocytes, are involved in the phagocytosis of apoptotic cells and microbes, such as bacteria and yeast. The phagocytic activity of the plasmatocytes plays a crucial role in development by clearing the embryo and pupa of apoptotic debris (Franc et al., 1996; Fujita et al., 2012; Kurant et al., 2008; Kurucz et al., 2007; Manaka et al., 2004; Nagaosa et al., 2011), but plasmatocytes are also important for an efficient immune response in the fly (Charroux and Royet, 2009; Defaye et al., 2009). Plasmatocytes remain phagocytically active during the whole life cycle of the fly, although the proportion of cells capable of phagocytosis decreases with age, a phenomenon which has been linked with immunosenescence (Horn et al., 2014; Mackenzie et al., 2011).

The first step of phagocytosis involves the recognition of harmful non-self from beneficial self. In *Drosophila*, several membrane-bound receptors with different ligand specificities have been associated with phagocytic activities. The receptors identified so far include the *Drosophila* Scavenger receptor class C homolog, type I (SR-CI), PGRP-LC, which is also involved in Imd signaling, as was discussed earlier, the EGF-repeat containing receptors Eater, NimrodC1 (NimC1) and Draper, the CD36 family receptors Croquemort and Peste, and the immunoglobulin superfamily receptor Dscam (Down syndrome cell adhesion molecule) (Kocks et al., 2005; Kurucz et al., 2007; Philips et al., 2005; Rämet et al., 2001; Rämet et al., 2002b; Stuart et al., 2005; Watson et al., 2005) (Figure 4). In *Drosophila*, NimC1, Eater and SR-CI are required for the efficient phagocytosis of both *Eschericia coli* and *Staphylococcus aureus* (Kocks et al., 2005; Kurucz et al., 2007; Rämet et al., 2001). However, it is unlikely that SR-CI plays a significant role in the phagocytosis of these bacteria, and the effect of NimC1 on the phagocytosis of *E. coli* is relatively modest (Kurucz et al., 2007; Rämet et al., 2001), although NimC1 does contribute considerably to the

phagocytosis of *S. aureus*. Instead, Eater seems to play a significant role in the phagocytosis of both *E. coli* and *S. aureus* and is possibly also involved in the phagocytosis of *E. faecalis* (Chung and Kocks, 2011; Nehme et al., 2011). In addition to Eater and NimC1, their family member Draper has also been associated with bacterial phagocytosis, although Draper was first discovered for its role in the phagocytosis of apoptotic particles (Cuttell et al., 2008; Hashimoto et al., 2009; Manaka et al., 2004). The *Drosophila* CD36 paralog Croquemort appears to be involved in the phagocytosis of *S. aureus*, although its influence is relatively minor; knock-down of Croquemort results in a 35 % reduction in the phagocytic ability of S2 cells (Stuart et al., 2005), but the receptor has also been linked to the phagocytosis of apoptotic particles (Franc et al., 1996). In addition to having a major role in the Imd signaling pathway, PGRP-LC has also a minor effect on the phagocytosis of *E. coli* (Rämet et al., 2002b). The other CD36 family receptor Peste has been implicated in the phagocytosis of the intracellular bacterium *Mycobacterium fortuitum*, but it is not involved in the phagocytosis of *S. aureus* and *E. coli* (Philips et al., 2005). Besides having a role in microbial phagocytosis, the phagocytic receptors Eater and dSR-CI have a major role in the uptake of dsRNA into *Drosophila* S2 cells (Ulvila et al., 2006).

A rather interesting player in the group of phagocytic receptors is Dscam, which has been reported to be involved in the phagocytosis of *E. coli*, and through alternative splicing, could theoretically be processed into more than 18,000 isoforms (Watson et al., 2005). The abundance of possible splice variants formed by Dscam resembles the somatic recombination of immunoglobulin production in vertebrates, suggesting that Dscam molecules might be specific for a variety of different ligands. Still, despite the immunoglobulin-like repertoire of possible Dscam isoforms, the presence of an adaptive immune system in the fruit fly is unlikely. Similarly, the actual functional importance of Dscam for the *Drosophila* immune system remains to be discovered (Armitage et al., 2014).

Opsonization, or the coating of microbes by antibodies or components of the complement system, can aid the phagocytosis of microbes by phagocytes in vertebrates. Opsonin-dependent phagocytosis is known to take place in mammals and may exist in the fruit fly as well. Especially, the complement-like TEPs (thio-esther containing protein) have been proposed to be capable of opsonization but also certain Dscam isoforms may function as opsonins (Lagueux et al., 2000; Stroschein-Stevenson et al., 2006; Watson et al., 2005). A relatively recent study also suggests that the Eater-dependent phagocytosis of Gram-negative bacteria might be aided by the coating of bacteria by AMPs or lysozymes (Chung and Kocks, 2012).

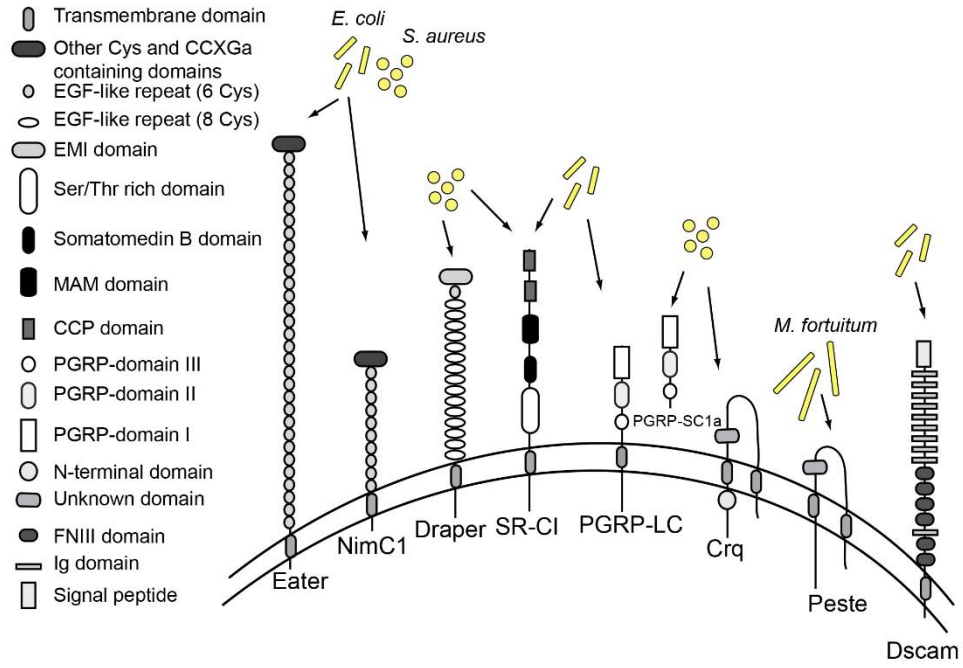

Figure 4. Receptors involved in *Drosophila* phagocytosis. Modified from Ulvila et al., 2011b.

Although several receptors involved in phagocytosis have been characterized, the knowledge of other key mediators and actors in phagocytosis has been limited. To this end, several large scale screens have been carried out identifying important players in microbial phagocytosis, but still many remain unidentified (Agaisse et al., 2005; Cheng et al., 2005; Derre et al., 2007; Garg and Wu, 2014; Garver et al., 2006; Koo et al., 2008; Pearson et al., 2003; Philips et al., 2005; Rämet et al., 2002b; Stroschein-Stevenson et al., 2006; Stuart et al., 2005; Ulvila et al., 2006). In addition, the significance of the phagocytosis of microbes for the host defense of the fruit fly has been unclear, but some reports show that it plays an important role in the battle against infections. Already in 1998, Braun et al. reported that *domino* mutants that have severely reduced hemocyte numbers, accumulate microbes in their body cavity and die younger than wild-type flies after a microbial infection, but still elicit a wild type-like AMP response (Braun et al., 1998). Moreover, *eater* null mutants are highly susceptible to bacterial infection although, like *domino* mutants, their ability to produce AMPs is not compromised (Kocks et al., 2005). Nevertheless, blocking phagocytosis by injecting polystyrene beads into wild-type flies did not increase their susceptibility towards an *E. coli* infection (Elrod-Erickson et al., 2000). More recent studies have shown that the ablation of hemocytes by apoptosis increased the

susceptibility of flies to *S. aureus* and *E. faecalis* infections, while the AMP response was not affected (Charroux and Royet, 2009; Defaye et al., 2009). Although contradicting evidence exists, these studies imply that phagocytosis functions together with the humoral aspects of *Drosophila* immunity to mount an effective host response against microbes.

2.4.3 Encapsulation in response to a wasp infection

In addition to bacteria, fungi and viruses, the immune system of fruit flies is also challenged by several wasp species that are natural parasites of fruit flies. Both ecto- and endoparasitic wasps attack fruit flies, and in nature even around 50% of fly larvae are infected by wasps (Carton et al., 2008; Fleury et al., 2004). Endoparasitic wasps lay eggs in 1st and early 2nd instar larvae, and the eggs develop inside the body cavity of the fruit fly larva. The oviposition of a wasp egg triggers a vigorous cellular immune reaction (Figure 5), the encapsulation response that seems to be unique to invertebrates, but is often functionally compared to granuloma formation in vertebrates. For encapsulation to occur, the wasp egg must be first recognized as non-self. The recognition of the egg then induces the activation of hemocytes and the formation of lamellocytes. The hemocytes migrate towards the egg, attach to it and to each other, and form a multilayered capsule around the egg to sequester it from the hemocoel. If the fly is successful in overcoming the parasitization, the wasp egg is killed inside the melanized capsule, most likely due to the release of cytotoxic free radicals (Carton et al., 2008; Russo et al., 1996). On the other hand, a successful parasitization leads to the death of the fruit fly larva as the parasite consumes the host by feeding on it, and eventually a wasp emerges out of the pupal case (Keebaugh and Schlenke, 2014).

Parasitization triggers changes in gene expression and hematopoiesis in fruit flies leading to an increase in the number of circulating hemocytes and formation of lamellocytes that specialize in encapsulation (Benassi et al., 2000; Crozatier et al., 2004; Krzemien et al., 2007; Lanot et al., 2001; Schlenke et al., 2007; Wertheim et al., 2005). The appearance of lamellocytes upon a wasp infection has been linked to the disruption of the lymph gland (Crozatier et al., 2004; Honti et al., 2010; Sorrentino et al., 2002), but also the sessile compartment has been shown to act as a pool for lamellocytes (Honti et al., 2010; Márkus et al., 2009). The sessile cells are mobilized from the subepidermal layer of the body cavity in response to a wasp infection,

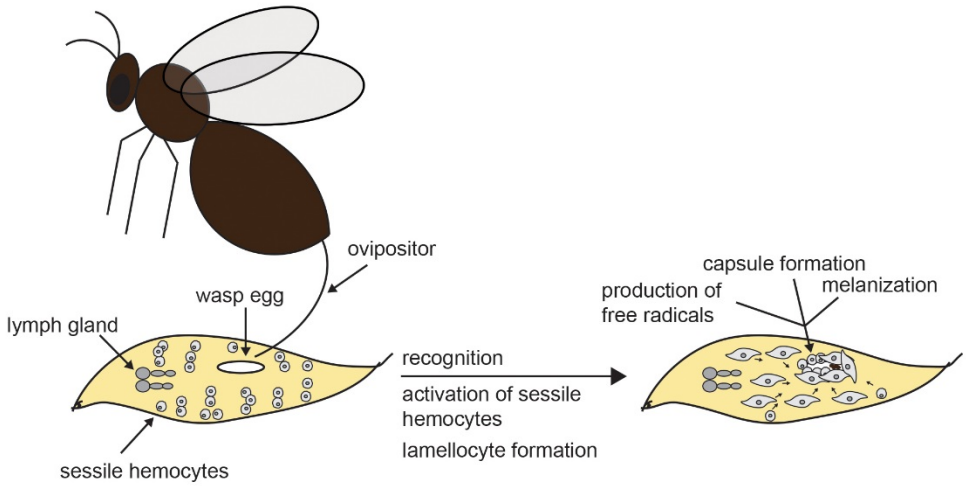

Figure 5. Overview of the encapsulation response triggered by wasp parasitism.

and the cells enter the circulation causing the disappearance of the banded pattern that is characteristic for the sessile compartment (Zettervall et al., 2004) (Figure 5). Encapsulation requires the action of all of the hemocyte types, but the role of lamellocytes is highlighted by *Drosophila subobscura*, which is devoid of lamellocytes and is therefore unable to encapsulate parasites (Eslin and Doury, 2006). As soon as the wasp egg is recognized by a mechanism still unknown, the formation of the capsule starts by circulating plasmatocytes and lamellocytes attaching to the wasp egg (Russo et al., 1996). Many aspects of the formation of the capsule are still not understood or are poorly characterized in comparison to the humoral elements of *Drosophila* immunity or the recognition molecules involved in phagocytosis. Nevertheless, several signaling pathways such as the JAK/STAT and Toll pathways, are known to be involved in the activation of hemocytes and the formation of lamellocytes (Sorrentino et al., 2004; Zettervall et al., 2004). Besides the increase in hemocyte number, changes in the cytoskeleton and adhesive properties are required for a proper capsule to form around the wasp egg. Studies done on mutants have shown that the Rho-family GTPases Rac1 and Rac2 are essential for a normal encapsulation response by controlling cell-to-cell adhesion and cell shape changes in hemocytes (Williams et al., 2005; Williams et al., 2006; Williams et al., 2007). Rac1 is also required for the increase in circulating hemocyte numbers upon a wasp infection (Williams et al., 2006; Zettervall et al., 2004). Microarrays have also shown the involvement of humoral factors and serine proteases in the encapsulation reaction (Schlenke et al., 2007; Wertheim et al., 2005). Serine proteases have been reported to

be required for the activation of prophenoloxidase into phenoloxidase, which takes part in the melanization of the inner layers of the capsule (Nappi et al., 2009).

Despite having an efficient immune reaction against parasitoids, the fruit fly is not always successful in eliminating the wasp larva. The constant arms race between the host and the parasite has led to several strategies for how the wasp can evade detection by the host immune system. These strategies include the coinjection of immunosuppressive components such as venom, polydnaviruses and virus-like particles (Asgari, 2006; Colinet et al., 2013; Strand and Burke, 2012). The composition of the wasp venom is not very well known, but the venom of *Leptopilina boulardi* contains at least the RhoGAP protein LbGAP that causes changes in the morphology of lamellocytes consequently inhibiting the encapsulation response (Colinet et al., 2007; Colinet et al., 2010; Labrosse et al., 2005; Labrosse et al., 2005), possibly by antagonizing the function of Rac1 and Rac2. The wasp venom also includes other components that interfere with the melanization reaction or induce changes in the surface proteins of lamellocytes inhibiting capsule formation (Colinet et al., 2011; Mortimer et al., 2012). In addition, the polydnaviruses injected by the wasps express their own genes in the host to ensure a successful parasitization by interfering with NF-κB signaling (Gueguen et al., 2013; Schmid et al., 2014).

3 Aims of the Study

Several large-scale or genome-wide *in vitro* and *in vivo* screens have been carried out in the fruit fly in order to identify genes that participate in the regulation of immune responses. As a result, many components of the *Drosophila* immune signaling pathways as well as genes involved in phagocytosis are already known. Growing evidence for the complexity and controlled regulation of the innate immune responses in *Drosophila* demonstrate that our knowledge of the components of the immune system is still relatively limited, and that many pieces in the puzzle remain to be characterized.

The specific aims of this study were:

1) To study the role of selected immunity-related genes previously identified in large-scale screens both *in vitro* and *in vivo* and to analyze their role in the host defense of the fruit fly

2) To analyze the significance of selected candidate genes in the phagocytic response of the fruit fly both *ex vivo* and *in vivo*.

3) To analyze the role of the infection-inducible gene, *edin*, in the humoral and cellular immune responses of the fruit fly.

4 Materials and Methods

4.1 S2 cell culture (II)

Drosophila macrophage-like S2 cells (Schneider, 1972) derived from late embryos were maintained in Schneider's insect cell culture medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% FBS (fetal bovine serum), 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma-Aldrich, St. Louis, MO, USA) at +25°C.

4.2 dsRNA synthesis (II)

Double-stranded RNA (dsRNA) synthesis was carried out using targeted primers (Table 2) and S2-cell complementary DNA (cDNA) as a template. A two-step PCR reaction was carried out. First, the target region was amplified by gene-specific primers and the resulting PCR product was used as a template for the second PCR reaction. The second reaction was carried out using nested gene-specific primers containing the T7 polymerase binding site in the 5' end of the primers (5'-GAATTAATACGACTCACTATAGGGAGA-3'). The T7 MegaScript RNA Polymerase kit (Ambion/Life Technologies, Carlsbad, CA, USA) was used to synthesize dsRNAs from the PCR products according to the manufacturer's instructions. *GFP* dsRNA was synthesized using the pMT/BiP/V5-His/GFP plasmid as a template.

4.3 Luciferase reporter assays (II)

Luciferase reporter assays were carried out to analyze the activity of the Imd, Toll and JAK/STAT pathways.

For the analysis of the Imd pathway, S2 cells were transfected with an *Attacin A* (*AttA)* (Tauszig et al., 2000) luciferase construct together with the FuGENE transfection reagent (Roche Applied Sciences, Basel, Switzerland). The *Actin5C-β-galactosidase* expression vector was used in all experiments to control for transfection

Table 2. Primer pairs used in dsRNA synthesis

Gene	Forward primer sequence (5'→3')	Reverse primer sequence (5'→3')	Product size (bp)
edin	GTT CTC CAA CAA GTG CGG	CAG AAA TGC CAG GTG CCC	345
	T7+ GGA ATA CTG ATC CTC G	T7+ CTT CGT AGT TGT TCC G	355
Cactus	GTT GAT AGA ACT GCT CCC	AGC AGC GGA GGC AGC AAC	659
	T7 + AGC GGA TGA TGT TGC	T7 + GAC TGC AGC TGC AGC	600
GFP	T7+ GCT CGG GAG ATC TCC	T7+ CTA GAC TCG AGC GGC	777
MyD88	GCC CTC GAT TTG TAT GCC	CGG CGT CTG CAG CTT GC	1604
	T7+ GCT GGC CAA GCA GAA GG	T7+ GGA ACG AGC CAA CTT GTC	766
Relish	CCA GCA CCA GTG GCT ATA GC	GCT CAT CGT TGC CCA TCA CC	916
	T7+ GCA AAC GGA CTT CGC	T7+ CTC ACG CTC TGT CTC	635
STAT	GAT GCA CTC GAC TGT GGG	CCC ATG GTT ACC GGG TAC	673
	T7+ CCA CGT GGT TAT GGC	T7+ GGA CCC CAG TGA TCT	604

efficiency and cell viability. The Imd pathway was activated by adding either heat-killed *E. coli* (strain BL21), *Serratia marcescens*, *Enterobacter cloacae*, peptidoglycan (PGN) (Sigma-Aldrich) or a pMT[*PGRP-LC*] construct to the cells 48 h post transfection. *Relish* and *GFP* dsRNAs were used as positive and negative controls, respectively.

Toll pathway activity was analyzed by transfecting S2 cells with a *Drocomycin* (*Drs)* luciferase reporter plasmid, and the pathway was activated with a constitutively active form of the Toll receptor, *Toll*^{10B} (Rosetto et al., 1995), or with a cleaved, active Spätzle ligand. *MyD88* dsRNA was used as a positive control and *GFP* dsRNA as a negative control. dsRNA targeted against the inhibitory protein Cactus was used as an additional positive control in the Spätzle-induced assay.

The activity of the JAK/STAT pathway was analyzed by transfecting the S2 cells with a *TurandotM* (*TotM*) luciferase reporter plasmid. The pathway was activated with a constitutively active form of the *Drosophila* JAK, hopscotch (hop). *STAT* and *GFP* dsRNAs were used as positive and negative controls, respectively.

The S2 cells were harvested 72 h post-transfection, pelleted and the pellets were lyzed in Passive Lysis Buffer (PLB; Promega, Madison, WI, USA) with the exception of *E. coli* -induced cells that were harvested 1 h, 4 h, 8 h and 24 h post-transfection. The cell lysates were centrifuged and the luciferase activity was measured from the supernatants with the Luminoskan Ascent luminometer (Thermo Labsystems, Waltham, MA, USA).

4.4 Western blotting (II)

S2 cells were transfected with *edin* which had been cloned into a heavy-metal inducible pMT-V5/HisA vector (Invitrogen/Life Technologies, Carlsbad, CA, USA). The expression of the pMT[*edin*]-V5 was induced with $CuSO_4$ 24 h post transfection. The cells were harvested, pelleted and lyzed 24 h after the addition of $CuSO_4$. 25 μg of the cell lysate and supernatant were electrophoresed on a 12 % NuPAGE Bis-Tris gel (Invitrogen/Life Technologies), blotted onto a nitrocellulose membrane, and detected by Western blotting using a mouse anti-V5 antibody (Invitrogen/Lifetechnologies) and a goat anti-mouse antibody HRP (horseradish peroxidase) conjugate (Molecular Probes). The ECL Plus Western blotting detection system (GE Healthcare Life Sciences, Little Chalfont, UK) was used for detection.

4.5 Microbial culture (I-II)

The *Listeria monocytogenes* strain 10403S (a gift from Professor Shoichiro Kurata), *Enterococcus faecalis*, the *Staphylococcus aureus* strain 29213 (ATCC, Manassas, VA, USA) and *Staphylococcus epidermidis* were cultured in brain-heart infusion (BHI) broth. *E. cloacae* (β12) (Boman et al., 1974) and *Micrococcus luteus* were cultured in Luria-Bertani (LB) broth supplemented with either 15 ng/ml of nalidixic acid (Sigma-Aldrich, St. Louis, MO, USA) or 100 μg/ml of streptomycin (Sigma-Aldrich), respectively. *E. coli* was cultured in LB supplemented with 100 μg/ml of ampicillin (Sigma-Aldrich). The baker's yeast *Saccharomyces cerevisiae* was grown overnight in YPDA medium (Gibco/Life Technologies, Carlsbad, CA, USA) supplemented with 10 μg/ml kanamycin at +30°C with shaking.

4.6 Colony forming unit assay (II)

4.6.1 Production of Edin in S2 cells

S2 cells were seeded onto 48-well plates in an antibiotic-free medium and transfected with 0.5 μg of the pMT[*edin*]-V5 plasmid or an empty plasmid. $CuSO_4$ was added to a final concentration of 300 μM 48 h post-transfection to induce the expression of the plasmid.

100 µl of fresh bacterial suspension (OD_{600nm}=0.33, ~1*10^6 bacterial/ml) was pelleted and the pellet was resuspended in 1 ml of Schneider's insect medium supplemented with 10% FBS. 50 µl of either *E. coli* or *S. aureus* was added to the S2 cells 24 h after the addition of $CuSO_4$ and incubated for 2 h at +25°C. The cells were harvested and serial dilutions of the suspension were made in sterile water. 20 µl droplets of each dilution were applied to LB (*E. coli*) or BHI (*S. aureus*) agar plates, and the plates were kept at +37°C overnight. The amount of colonies were counted the next day and used to determine the colony forming unit (cfu) per milliliter.

4.6.2 Synthetic forms of Edin

Two synthetic forms of Edin (N-terminal and C-terminal peptides) were ordered from Peptide 2.0. (Chantilly, VA, USA). The peptides were dissolved in H_2O according to the manufacturer's instructions. The amino acid sequences for the peptides were SYRQP YPEEF QTSPE QLLQV APLV (N-terminal fragment) and SPEGG SVVVT ASKDN QVGRE ASVQY NHNLY SSGDG RGSID AYAQA SRNFD YNRNN YEGGI RGTWHF (C-terminal fragment) (Figure 6).

An overnight culture of bacteria was pelleted and resuspended in 5% DMSO. 5 µl of *E. coli*, *E. cloacae*, *L. monocytogenes*, and *E. faecalis* suspensions were added onto 96-well plates containing the synthetic Edin at different concentrations. The samples were incubated for 2 h at +25°C, after which serial dilutions were made as described in 4.6.1. The diluted samples were plated as in 4.6.1 and the bacterial colonies were counted. Lysozyme and Cecropin A (Sigma-Aldrich, St. Louis, MO, USA) were used as positive controls for Gram-positive and Gram-negative bacteria, respectively.

MFSNKCGILI LVSCCLVTIV A*SYRQPYPEE FQTSPEQLLQ VAPLV*RRAR**S PEGGSVVVTA SKDNQVGREA SVQYNHNLYS SGDGRGSIDA YAQASRNFDY NRNNYEGGIR GTWHF**

Figure 6. The amino acid sequence of Edin depicting the N-terminal signal sequence (underlined) and the amino acid sequences of the N-terminal (italics) and C-terminal (bold) synthetic peptides.

4.7 Binding assay (II)

The assay for analyzing the binding properties of Edin was carried out as described elsewhere (Rämet et al., 2001) with some modifications. S2 cells were seeded onto 24-well plates and transfected with 0.5 μg of the pMT[*edin*]-V5 plasmid or an empty pMT-V5 plasmid. $CuSO_4$ was added 48 h post-transfection to a final concentration of 500 μM. The cells were harvested 24 h later, and the supernatant was collected. One ml of a microbial culture grown overnight was pelleted by centrifugation and the pellet was washed 5 times with 1 x PBS. 500 μl of the medium containing either the pMT[*edin*]-V5 or the empty pMT-V5 was added onto the washed microbial pellets or onto the latex bead controls treated with 0.4 M N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (EDC) and 0.1 M N-hydroxysuccinimide (NHS) and coated with BSA (10 mg/ml in PBS, pH 7.4). The samples were incubated in an end-to-end rotator for 1 h at +4°C. Thereafter, the samples were centrifuged and the pellet was washed 5 times with 1 x PBS and the pellet was resuspended in 20 μl of PBS. Next, SDS-PAGE sample buffer was added and the samples were boiled for 10 min to detach bound Edin from the microbial cells. The samples were centrifuged and 30 μl of the supernatant was loaded onto a 12 % NuPAGE BisTris gel and Western blotting was carried out as in 4.4.

4.8 Total RNA extraction (I-III)

RNA was extracted from S2 cells, adult flies, larvae or fat bodies using either the TRIsure (Bioline, London, UK) or the TRIzol reagent (Life Technologies, Carlsbad, CA, USA). The samples were homogenized in the reagent and total RNAs were extracted according to the manufacturer's instructions.

4.9 Quantitative RT-PCR (I-III)

Quantitative real-time PCR (qRT-PCR) was performed using the Quantitect SYBR Green RT-PCR kit (Qiagen) and the ABI7000 instrument (Applied BioSystems/Life Technologies) or with the iSript One-Step RT-PCR kit with SYBR Green (Bio-Rad) and the Bio-Rad CFX96 instrument (Bio-Rad) according to the manufacturer's instructions. Results were analysed with the ABI7000 system SDS software version 1.2.3 (Applied Biosystems) or with the Bio-Rad CFX Manager software version 1.6

(Bio-Rad), respectively. *Actin5C* or *rpl32* were used as housekeeping genes. The primers used in qRT-PCR are listed in Table 3.

Table 3. Primer pairs used in quantitative PCR

Gene	Forward primer sequence (5'→3')	Reverse primer sequence (5'→3')	Product size (bp)
14-3-3ζ	CTAC GAA GTT TTG GGA CTT	CCC TTG CTA ATG TCA AA	192
Actin5C	CGA AGA AGT TGC TGC TCT GG	AGA ACG ATA CCG GTG GTA CG	453
Attacin A	TTT GGC CTA CAA CAA TGC TG	GCT TCT GGT TGG CAA ACG	374
Attacin B	CAG TTC CCA CAA CAG GAC C	CTC CTG CTG GAA GAC ATC C	272
Attacin C	CAT CGT TGG CGT ACT TGG C	TTG CTG GAA GCT ATC CCG C	353
Cecropin A1	CGT CGC TCT CAT TCT GGC	GTT GCG GCG ACA TTG GC	153
Cecropin B	TTG TGG CAC TCA TCC TGG	TCC GAG GAC CTG GAT TGA	122
Diptericin B	GAC TGG CTT GTG CCT TC	CCT GAA GGT ATA CAC TCC	326
Drosocin	TTC CTG CTG CTT GCT TGC G	TGG CAG CTT GAG TCA GGT G	148
Drosomycin	GTT CGC CCT CTT CGC TG	GCA TCC TTC GCA CCA GC	190
Edin	CTC GTG TCC TGC TGT CTG	GCC TTC GTA GTT GTT CCG	291
Edin	CAA CAA GTG CGG AAT ACT G	TCA GAA ATG CCA GGT GC	340
Rpl32	TTC TGC ATG AGC AGG ACC TC	GGT TAC GGA TCG AAC AAG CG	101

4.10 *In vivo* experiments (I-III)

4.10.1 *Drosophila* stocks (I-III)

Flies were kept at +25°C on a regular mashed potato, syrup and yeast diet.

The *edin* RNAi lines #14289 and #109528 (hereafter called $edin^{109528}$ and $edin^{14289}$), the 14-3-3ζ RNAi line (#48725) and the *Eater* RNAi line (#4301) were obtained from the Vienna *Drosophila* resource center (Dietzl et al., 2007). *14-3-3ζ* RNAi lines (*17870-R1* and *17870-R2*), *Abi* RNAi lines (*9749-R3*), and *cpa* RNAi lines (*10540-R2*) were obtained from the NIG-Fly Stock Center (National Institute of Genetics, Mishima, Shizuoka, Japan). Transgenic *edin* overexpression flies were generated in

the Umeå Fly and Worm facility by microinjecting a *pUAST-edin* construct into the *Relish*^{E20} background (hereafter called *UAS-edin,Rel*^{E20}). The deficiency lines *Df(3R)Tl-I/TM3,P{ActGFP}JMR2,Ser1* and *Df(3R)D605/TM3,P{ActGFP}JMR2,Ser1* were used to create the *eater* null flies (Kocks et al., 2005).

The binary UAS-GAL4 system (Brand and Perrimon, 1993) was used in this study to drive the expression of the RNAi or overexpression constructs in different target tissues. The *Hemolectin*$^{\Delta}$*-GAL4* (*Hml*$^{\Delta}$*-GAL4*) and *Hemese-GAL4* (*He-GAL4*) lines were used as hemocyte-specific drivers. *Hml*$^{\Delta}$*-GAL4* is expressed in the majority of plasmatocytes and crystal cells (Goto et al., 2003) as well as in the lymph glands, whereas *He-GAL4* is expressed in most circulating hemocytes, but not in the lymph glands (Zettervall et al., 2004). The combined *Hml*$^{\Delta}$*;Hemese-GAL4* driver is expressed in essentially all hemocytes (*Hml*$^{\Delta}$*-GAL; He-GAL4*) (Schmid et al., 2014). The *C564-GAL4* driver was obtained from Bruno Lemaitre's laboratory (Global Health Institute, EPFL, Switzerland) and is expressed in the fat body, lymph glands, salivary glands, gut and brain, but not in hemocytes (Harrison et al., 1995). The *Fatbody-GAL4* (*Fb-GAL)* driver targets the expression strongly to the fat body, although also some ectopic expression is detected in other tissues, but not in the hemocytes (Schmid et al., 2014). The *Actin5C-GAL4/CyO* and *Daughterless-GAL4* (*Da-GAL4)* drivers are ubiquitously expressed.

The hemocyte reporter lines *eaterGFP* and *MSNF9mo-mCherry* (*msnCherry)* were obtained from Robert Schulz's laboratory. The lines were crossed together to obtain the *eaterGFP,msnCherry* reporter line.

Canton S and *w*^{1118} wild-type lines and *Relish*^{E20} null mutants were used as controls in the experiments.

4.10.2 Lifespan experiments (II)

UAS-edin,Rel^{E20} flies were crossed with the *C564-GAL4*, *Actin5C-GAL4/CyO* (*Act5C-GAL4/CyO*) and *Daughterless-GAL4* (*Da-GAL4*) driver lines. The lifespan of the resulting offspring was monitored at +25°C. *Relish*^{E20} flies crossed with the above-mentioned driver lines were used as controls. The flies were transferred into fresh vials containing 5 ml of standard fly food twice a week and their survival was monitored. Males and females were kept in separate vials, 10 to 20 flies per vial.

4.10.3 Phagocytosis assay with primary hemocytes (I)

The *14-3-3ζ* (#48725), *cpa* (*10540-R2*) and *Abi* (*9749-R3*) RNAi fly lines were crossed with the *Hml*Δ*-GAL4* driver line. w^{1118} flies crossed with the RNAi lines or the *Hml*Δ*-GAL4* driver line were used as negative controls. As a positive control for defective phagocytosis, we used the *Eater* RNAi line (#4301) crossed with *Hml*Δ*-GAL4*. The larval hemocytes from the resulting offspring were collected for the experiments. First, late wandering 3rd instar larvae were disinfected with a 5% sodium hypochlorite solution and then washed three times in H_2O. Eight to ten larvae per sample were bled into 1 ml of ice-cold fresh Schneider's medium (Sigma-Aldrich) without any supplements in 48-well plates. The larval hemocytes were allowed to set for 10-20 min and then 3 x 10^6 FITC-labeled, heat-killed bacteria were added and the samples were centrifuged briefly. The hemocytes were left to phagocytose for 10 min at +25°C. The samples were returned to ice and the cells were fixed with 2% glutaraldehyde at room temperature. The extracellular fluorescence was quenched with a trypan blue solution. Microscopy and imaging were performed using the Olympus IX71 inverted fluorescent microscope with the F-view soft imaging system and the QCapture Pro 6.0. software. The phagocytic index represents the number of internalized bacteria/cell.

4.10.4 *In vivo* phagocytosis assay (I)

The crosses made for the analysis of the phagocytic capability of flies *in vivo* were the following: *14-3-3ζ* RNAi flies (#48725) and w^{1118} flies were crossed with the *Hml*Δ*-GAL4* driver line and the *14-3-3ζ* RNAi line was crossed with w^{1118} as a control. *Eater* null flies which are deficient in phagocytosing *E. coli* were used as a positive control for impaired phagocytosis.

pHrodo-conjugated heat-killed *E. coli* (Invitrogen/Life Technologies, Carlsbad, CA, USA) were resuspended in sterile PBS to a final concentration of 4 mg/ml. The suspension was vortexed, sonicated and pulled through a 30-G needle to avoid aggregation of the particles. 25 nl of the suspension was injected into 1-week-old healthy male flies using the PV839 Pneumatic PicoPump microinjector (WPI, Sarasota, FL, USA). As a control, flies were injected with 70 nl of sterile PBS or surfactant-free carboxylate-modified latex beads with a diameter of 0.3 μm (Invitrogen/Life Technologies) 24 hours prior to injection with pHrodo-conjugated *E. coli* (Elrod-Erickson et al., 2000). The flies were incubated for 30 min at room temperature apart from temperature-control flies, which were incubated at +4°C.

Next, the flies were anesthetized with FlyNap (Carolina Biological Supply Co., Burlington, NC, USA) and mounted on agarose plates. Imaging was carried out with a Zeiss Lumar.V12 stereomicroscope with an AxioCam MRm camera and the AxioVision Rel. 4.8. software. The images were quantified with ImageJ by measuring the area of pixels with a gray value of ≥115 and by measuring their mean gray value within the fly abdomen. The phagocytic index represents the area multiplied by the mean gray value. Statistical analyses were performed using one-way ANOVA and the statistical significance was set at $p < 0.05$.

4.10.5 Bacterial infection experiments (I-II)

Infection experiments were carried out with one-week-old healthy flies by pricking the flies into the thorax area with a thin tungsten needle dipped into a bacterial concentrate of either *E. coli*, *E. cloacae, E. faecalis*, *L. monocytogenes* or *S. aureus*. The flies infected with *E. faecalis* were pricked with *M. luteus* 24 hours prior to the *E. faecalis* infection in order to activate the Toll pathway. The bacteria were grown overnight on culture plates with the following exceptions: *S. aureus* and *E. cloacae* were cultured in BHI or LB broth to an OD_{600nm} of 1.7 and 2.0, respectively. The bacterial suspensions were pelleted by centrifugation, and a 1:10 dilution in sterile glycerol was prepared.

Statistical analyses were carried out using the Log Rank analysis.

4.10.6 Wasp infection experiments (III)

Ten *GAL4*-driver virgin females were crossed with 5 RNAi males and allowed to lay eggs at room temperature for 24 h. w^{1118} flies, *GAL4*-driver virgin females crossed with w^{1118} males and w^{1118} virgin females crossed with RNAi males were used as controls. The crosses were transferred daily into fresh vials and the vials containing the eggs were transferred to +29°C. The resulting larvae from the crosses were infected at room temperature with 20 female and 10 male wasps of the *Leptopilina boulardi* strain G486 on the third day after egg-laying. The wasps were removed after 2 h and the larvae were returned back to +29°C.

The encapsulation activity of the larvae was assayed 27-29 h after infection and the killing ability 48-50 hours after wasp infection. The egg was considered encapsulated when melanin was found on it. When analyzing the killing ability, the wasp was scored as killed if the larva had managed to encapsulate the wasp egg and

no living wasp was found in the hemolymph. The wasp was considered living if a living wasp larva was present in the hemolymph with or without traces of a melanized capsule.

4.10.7 Flow cytometry of larval hemocytes (III)

The hemocyte reporter line *eaterGFP,msnCherry* was used to quantify the plasmatocytes and lamellocytes in wasp infected fruit fly larvae. Ten *eaterGFP,msnCherry;Fb-GAL4* virgin females were crossed with five *eaterGFP,msnCherry;edin*^{109528} males to drive the expression of the *edin* RNAi construct in the fat body. *eaterGFP,msnCherry* crossed with *eaterGFP,msnCherry;Fb-GAL4* or *eaterGFP,msnCherry;edin*^{109528} were used as controls. The homozygous *eaterGFP,msnCherry* reporter line alone was also used as a control. One larva represents one sample.

The larvae were infected with the *L. boulardi* strain G486 and the hemocytes were bled into 100 µl of PBS with 8% BSA 27-29 hours post infection. Flow cytometry was carried out to detect *eater-GFP* positive and *msnCherry*-positive cells 27-29 h after wasp infection. Uninfected, age-matched larvae were used as controls. The samples were run using the Accuri C6 flow cytometer (BD Biosciences, San Jose, CA, USA) and the data was analyzed using the BD Accuri C6 software. The most forward-scattered cells were selected to represent living cells. The gating strategy was based on the unpublished results by I. Anderl & L. Vesala et al., which is explained in more detail in Figure S1 in original publication III.

4.10.8 Imaging of *Drosophila* hemocytes (III)

Imaging of *Drosophila* hemocytes was carried out using the *eaterGFP,msnCherry* reporter line and the same crosses were made as in 4.10.7. For imaging purposes, the larvae were collected 48-50 h after wasp infection, washed three times in H_2O and bled into 1 x PBS. Uninfected controls of the same age were treated similarly. The hemocytes were allowed to adhere to the glass surface microscope slides for 30 min and were then fixed with 3.7% paraformaldehyde for 5 minutes. The samples were washed with 1 x PBS and mounted with the Prolong Gold Anti-Fade reagent with DAPI (Molecular Probes/ Life Technologies). The imaging was carried out using the Zeiss AxioImager.M2 microscope with the Zeiss AxioCam and the Zen Blue 2011 software.

4.10.9 Live imaging of *Drosophila* larvae (III)

Live imaging of 3rd instar fruit fly larvae was carried out 27-29 h after wasp infection. The larvae were washed three times in H_2O, dried in tissue paper and embedded on microscope slides in a drop of ice-cold glycerol. The larvae were immobilized at -20°C before imaging. The imaging was performed using a Zeiss AxioImager.M2 microscope with the Zeiss AxioCam and the Zeiss ApoTome.2 and the Zen Blue 2011 software.

5 Summary of the Results

5.1 *In vivo* analysis of Abi, cpa and 14-3-3ζ as potential regulators of phagocytosis (I)

Phagocytosis is an evolutionarily conserved innate immune response that involves the uptake of microorganisms by immune cells. In the fruit fly, phagocytic plasmatocytes are responsible for the phagocytosis of microbes and apoptotic cells. The fruit fly is a powerful model organism for studying the mechanisms of phagocytosis, because these events are evolutionarily conserved and because several relevant genetic tools are available for *Drosophila* studies. Several large-scale screens have been carried out to identify key mediators and actors in the phagocytosis of microbes. These studies have revealed that phagocytosis involves a wide variety of molecules that function in, for example, the remodeling of the actin cytoskeleton, in endocytic trafficking and in signaling (Agaisse et al., 2005; Cheng et al., 2005; Derre et al., 2007; Garg and Wu, 2014; Garver et al., 2006; Koo et al., 2008; Pearson et al., 2003; Philips et al., 2005; Rämet et al., 2002b; Stroschein-Stevenson et al., 2006; Stuart et al., 2005). In original communication I, a flow cytometry-based RNAi screen of 3,048 dsRNAs was carried out to identify genes involved in the phagocytosis of *E. coli* in *Drosophila* S2 cells. The hits from the initial screen were analyzed with new dsRNAs to determine the effect of the genes on the phagocytosis of both *E. coli* and *S. aureus*. Of the six genes that had an effect on the phagocytosis of both bacteria, *14-3-3ζ*, *Abelson interacting protein* (Abi) and *capping protein* α (cpa) were found to have evolutionarily conserved roles in phagocytosis, as an siRNA (short interfering RNA) treatment of their mouse homologues in RAW 264.7 cells resulted in a decrease in the phagocytic ability of the cells.

The three genes that showed evolutionary conservation were subjected to further *in vivo* characterization using RNAi flies and tissue-specific knock-down experiments. The functional significance of 14-3-3ζ, cpa and Abi in phagocytosis was first studied in *Drosophila* larvae using an *ex vivo* phagocytosis assay. *14-3-3ζ*, *cpa* and *Abi* were silenced in larval hemocytes using the hemocyte-specific *Hml*$^{\Delta}$*-GAL4* driver, and the ability of the larval hemocytes to internalize heat-killed FITC-labeled *E. coli* was analyzed. Hemocyte-specific knock down of *14-3-3 ζ*, *cpa* and *Abi* resulted in a

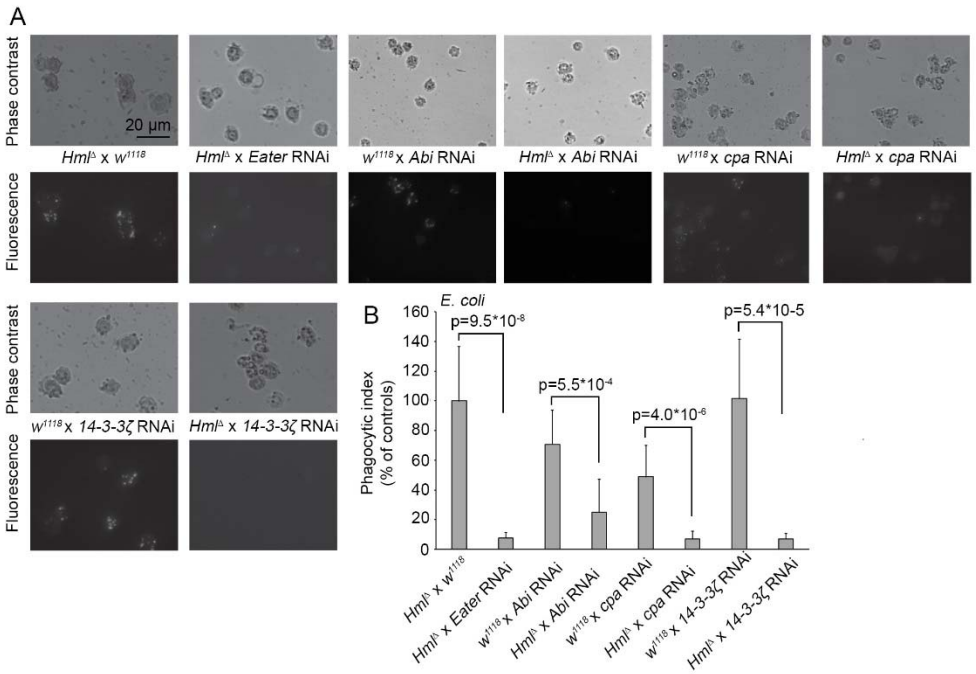

Figure 7. Abi, cpa and 14-3-3ζ are required for efficient phagocytosis *ex vivo*. (A) Representative microscope images of primary hemocytes that have phagocytosed heat-killed FITC-labeled *E. coli.* (B) Quantification of the phagocytocic activity of larval hemocytes. *Hml$^{\Delta}$-GAL4 x w^{1118}* flies were used as a wild-type control and *Eater* RNAi flies were used as a positive control for defective phagocytosis. Modified from Ulvila et al., 2011a (published with permission).

significant decrease in the phagocytic ability of the hemocytes, validating the original *in vitro* finding (Figure 7; Figure 2 in I).

Because 14-3-3ζ had not been previously studied in the context of phagocytosis, we chose to characterize it further *in vivo*. The phagocytic ability of the *14-3-3ζ* RNAi flies was analyzed *in vivo* by injecting the flies with pHrodo-conjugated heat-killed *E. coli* and performing fluorescent microscopy. The pHrodo particles do not fluoresce in neutral pH, but start fluorescing in acidic pH, such as in the phagolysomes, removing the need for the quenching of the extracellular fluorescence. *14-3-3ζ* RNAi in hemocytes resulted in a decrease in the amount of fluorescence in the body cavity, indicating impaired phagocytic ability (Figure 8; Figure 3 in I). When the phagocytic index of *14-3-3ζ* RNAi flies was quantified based on the fluorescent area observed in the flies, a greater than 50% decrease in the phagocytosis of pHrodo-conjugated heat-killed *E. coli*, compared to controls, was observed (Figure 3K in I). These data

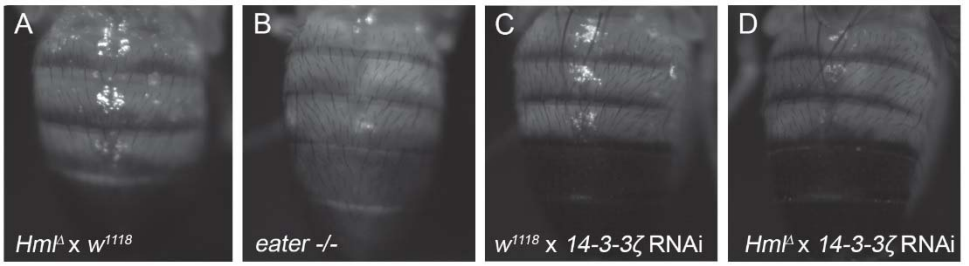

Figure 8. 14-3-3ζ is required for normal phagocytosis *in vivo*. (A-D) Representative images of the indicated crosses showing the phagocytosis of pHrodo-conjugated *E. coli*. Modified from Ulvila et al. 2011a (published with permission).

indicate that 14-3-3ζ is required for the normal phagocytosis of heat-killed *E. coli* in *Drosophila in vivo*.

5.1.1 Role of 14-3-3ζ in the resistance against bacterial infections (I)

To validate the role of 14-3-3ζ in immunity, we carried out a survival experiment with *14-3-3ζ* RNAi flies using an *S. aureus* infection model, because it has been previously shown that phagocytosis is an important determinant in the resistance against *S. aureus* in *Drosophila* (Charroux and Royet, 2009; Defaye et al., 2009). Two different *14-3-3ζ* RNAi lines were used and again, the RNAi was targeted to the hemocytes using the *Hml*$^{\Delta}$*-GAL4* driver. With both RNAi constructs, knocking down 14-3-3ζ in the hemocytes rendered the flies significantly more susceptible to an *S. aureus* infection in comparison to controls (Figure 9A). The *14-3-3ζ* RNAi flies did not succumb to an *E. cloacae* infection, excluding the possibility that the flies would have died because of a general deficiency in their immune system (Figure 5 B in I) (Charroux and Royet, 2009; Defaye et al., 2009). In addition, knocking down *14-3-3ζ* in the hemocytes did not affect AMP production in the flies, confirming that the flies have a normal humoral immune response (Figure 9B; Supplementary Figure 2 in I). These results indicate that 14-3-3ζ-dependent phagocytosis is required for an efficient host defense against bacteria.

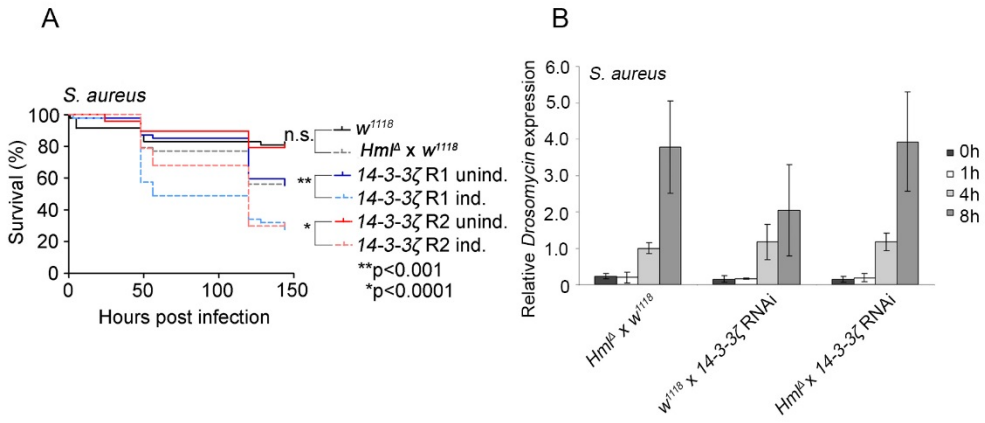

Figure 9. Knocking down *14-3-3ζ* in the hemocytes sensitizes the flies to an *S. aureus* infection but does not affect AMP production. (A) Survival of *14-3-3ζ* RNAi flies after an *S. aureus* infection. unind= uninduced (crossed with w^{1118}), ind= induced (crossed with *Hml*$^{\Delta}$*-GAL4*). (B) *Drosomycin* expression in *14-3-3ζ* RNAi flies after an *S. aureus* infection. The levels of other tested AMPs also remained unaffected by the *14-3-3ζ* RNAi. Modified from Ulvila et al., 2011a (published with permission).

5.2 *Edin* as a gene induced by bacterial infection (II)

In fruit flies, the expression of many genes is turned on in response to an immune-challenge (Irving et al., 2001; De Gregorio et al., 2001), and the change in the transcriptional status depends both on the type and on the site of the infection. Previously, our laboratory carried out a large-scale screen in S2 cells to study which genes were upregulated in response to an *E. coli* infection (Valanne et al., 2007). Among the most highly induced genes were AMPs and a novel immunity-related gene *edin* (CG32185), which was selected for further characterization in original publication II. Edin is a short secreted peptide of 115 amino acids with an N-terminal signal sequence, and its expression was rapidly and strongly induced after a Gram-negative bacterial infection (Figure 10A; Table 1 and Figure 1B in II). The expression pattern of *edin* resembled the expression pattern of known *AMP* genes and its expression seemed to be dependent on the Imd pathway, because the expression of *edin* was completely abolished in S2 cells when the transcription factor Relish was knocked down (Valanne et al., 2007). This observation was confirmed by a qRT-PCR analysis of S2 cells that were activated by heat-killed *E. coli* (Imd pathway) or an active form of the Toll receptor, $Toll^{10B}$ (Toll pathway). In this experimental setting, the expression of *edin* was induced by 8-fold with heat-killed *E. coli*, whereas

Toll^{10B} did not induce the expression of *edin* in the cells (Figure 10B). These results together show that the expression of *edin* is mediated via the Imd pathway *in vitro*.

To validate the *in vitro* results, we analyzed the expression pattern of *edin in vivo* using adult flies. Wild-type *Canton S* flies and *Relish*^{E20} null mutants were infected with *E. cloacae* and the transcript levels of *edin* were analyzed by RT-PCR and agarose gel electrophoresis. In line with the *in vitro* data, *edin* was not expressed in the absence of an infection, but it was rapidly upregulated upon a bacterial infection in *Canton S* flies (Figure 1C in II). In addition, *Relish*^{E20} mutants showed no induction of *edin*. These data together suggest that *edin* is induced in response to a Gram-negative bacterial infection in a Relish-dependent manner. In summary, our data show that *edin* is an infection-inducible gene, and that its expression can be induced by a Gram-negative bacterial infection. Knocking down the Imd-pathway transcription factor Relish abolished the expression of *edin* both *in vitro* and *in vivo* indicating that upon a bacterial infection, the expression of *edin* is regulated via the Imd pathway.

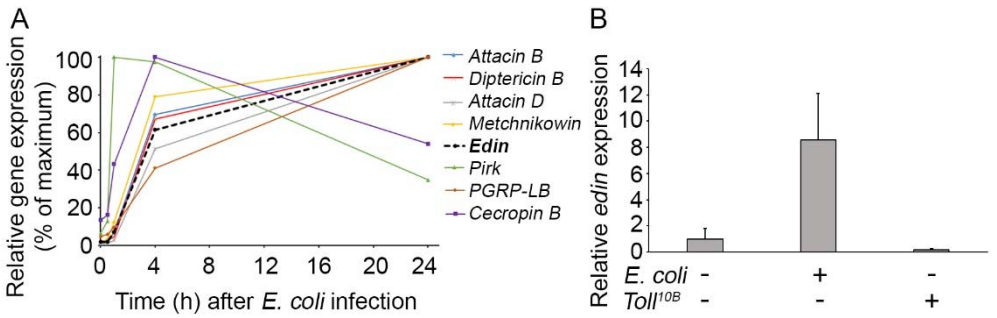

Figure 10. (A) Relative induction profiles of genes induced upon an *E. coli* infection in S2 cells. The graph represent the eight most induced genes collected from Valanne et al., 2007. (B) The expression of *edin* is induced by an infection and mediated via the Imd pathway *in vitro*. The expression of *edin* is Imd-pathway dependent *in vitro* and is not induced by activation of the Toll pathway. The Imd pathway was induced by *E. coli* and the Toll pathway by *Toll*^{10B} and the expression levels of *edin* were determined with qRT-PCR.

5.2.1 Antimicrobial properties of Edin (II)

Because Edin is a small, secreted peptide, which is induced upon infection and which shares many similarities with known AMPs, we hypothesized that it might be a novel antimicrobial peptide. To this end, we carried out several *in vitro* and *in vivo* experiments to study Edin's antimicrobial properties. First, we studied *in vitro*, whether Edin inhibited the growth of bacteria by overexpressing *edin* in S2 cells and incubating the Edin-containing medium with *E. coli* and *S. aureus*. In this experimental setting, Edin had no effect on bacterial growth (Figure 6A-B in II). We also failed to see any bacteriosidic or bacteriostatic properties when synthetic forms of Edin (See Figure 6 in chapter 4.6.2. for the amino acid sequence of the peptides) were incubated with *E. coli*, *E. cloacae*, *L. monocytogenes* and *E. faecalis* (Figure 6C-J in II). In addition, the synthetic peptides showed no synergistic inhibitory effects with either Lysozyme or Cecropin A that were used as positive controls in the experiments (Figure 6C-J in II).

We also tested the bacterial binding and opsonization properties of Edin by producing Edin in S2 cells and incubating the Edin-containing medium with living Gram-negative (*E. coli* and *S. marcescens*) and Gram-positive bacteria (*M. luteus*, *S. aureus*, *S. epidermidis*, *E. faecalis* and *L. monocytogenes*), as well as with the baker's yeast *S. cerevisiae*. Our results showed that Edin was not able to bind the tested Gram-negative bacteria and that *S. cerevisiae* and Gram-positive bacteria were bound only very weakly (Figure 3 in II). However, in comparison to the latex beads that were used as a positive control and to the control sample containing only Edin, the binding of Gram-positive bacteria and the baker's yeast was not significant indicating that Edin did not strongly bind any of the tested microbes (Figure 3 in II).

Despite the absence of antimicrobial properties *in vitro*, we examined the antimicrobial properties of Edin *in vivo* by using flies overexpressing *edin*. Gordon et al. reported that overexpressing *edin* reduced the lifespan of flies (Gordon et al., 2008), so we first followed the survival of flies overexpressing *edin* in the absence of an infection to evaluate whether overexpressing *edin* had detrimental effects on the lifespan of the flies. Although the overexpression of *edin* was driven with the ubiquitously expressed drivers *Act5C-GAL4/CyO*, *C564-GAL4* and *Da-GAL4*, no effect on lifespan was observed (Figure 7A in II). In addition, the ubiquitous overexpression of *edin* did not affect the survival of the flies, as equal amounts of *UAS-edin,Rel^{E20}/Act5C-GAL4* and *UAS-edin,Rel^{E20}/CyO* flies eclosed from the crosses (Figure 7B in II).

To study the antimicrobial properties of Edin in the context of a septic infection, we examined the effect of overexpressing *edin* in a *Relish*^{E20} mutant background and in a heterozygous *Relish*^{E20} mutant background. In the *Relish*^{E20} mutant flies, the production of AMPs via the Imd pathway is impaired, sensitizing the flies to bacterial infections (Hedengren et al., 1999), but in the heterozygous background, the flies produce AMPs at normal levels. If *edin* had antimicrobial properties *in vivo*, overexpressing *edin* in the sensitized homozygous mutant background could rescue the flies from succumbing to the bacterial infection, since the overexpression of a single AMP can restore the antimicrobial activity of the flies (Tzou et al., 2002). The *UAS-edin,Rel*^{E20} flies crossed with the *C564-GAL4;Rel*^{E20} driver were infected with two Gram-positive bacteria, *E faecalis* and *L. monocytogenes* that has a DAP-type peptidoglycan, as well as with the Gram-negative bacterium *E. cloacae*. The *C564-GAL* driver targets the expression of the construct to the fat body, the lymph glands, the salivary glands, the gut and the brain. In the homozygous *Relish*^{E20} mutant background, the overexpression of *edin* did not rescue the flies from succumbing to the infection with any of the tested bacteria (Figure 7C-E in II) indicating that either Edin does not have antimicrobial properties or that the expression of *edin* alone is not sufficient to restore the antimicrobial activity of the *Relish*^{E20} mutants. Similarly, when the effect of Edin was studied in the heterozygous *Relish*^{E20} mutant background, overexpressing *edin* did not positively affect the survival of the flies, and the flies expressing *edin* succumbed to the bacterial infections as fast as the control lines (Figure 7F-H in II) denoting that Edin does not have any obvious antimicrobial properties against Gram-negative or Gram-positive bacteria.

5.2.2 Edin in the modulation of immune signaling pathways (II)

The regulation of immune responses is under tight control in the fruit fly. The Imd, Toll and JAK/STAT pathways are major contributors to the immune signaling, and they are involved in such events as AMP production, hemocyte differentiation and wound healing (reviewed in Myllymäki et al., 2014; Myllymäki and Rämet, 2014; Valanne et al., 2011). To study the importance of Edin in the regulation of these pathways, we knocked down *edin* in S2 cells and analyzed the effects with a luciferase reporter assay. With the assay, the luciferase activity can be used to measure the transcriptional activity of the cells transfected with a reporter construct containing the *luciferase* open reading frame under the control of a promoter of interest. In this study, we used *Attacin A* (*AttA*), *Drosomycin* (*Drs*) and *Turandot M* (*TotM*) -luciferase

reporter constructs to analyze the transcriptional activity of the Imd, Toll and JAK/STAT pathways, respectively. The luciferase activity can be quantified from S2 cells with a luminometer, and the luciferase produced by the construct corresponds to the transcriptional activity of the promoter. The S2 cells were transfected with the luciferase reporters together with *edin* dsRNA and negative and positive control dsRNAs. *GFP* dsRNA was used as a negative control in all of the experiments, and the efficiency of the *edin* RNAi was validated with qRT-PCR (Figure 4A in II). Knocking down *edin* in S2 cells had no significant effect on the *Drs*-luciferase or *TotM*-luciferase mediated Toll or JAK/STAT pathway activities (Figure 4D-F in II), whereas the *edin* RNAi showed little or no effect on the Imd-pathway activity, the only trend being a small reduction in the *AttA*-luciferase activity at the 24 h time point (Figure 4B in II). Because of the small reduction at the 24 h time point, the role of Edin in the modulation of the Imd pathway was studied further by inducing the activity of the pathway with different pathway elicitors, namely heat-killed *E. cloacae* and heat-killed *S. marcescens*, PGN and PGRP-LC, but knocking down *edin* had no effect on the activity of the Imd pathway in this experimental setting (Figure 4C in II).

Similar results were obtained *in vivo*, when we examined the role of Edin in the regulation of the production of AMPs mediated by the Imd pathway. *Edin* overexpression and RNAi were driven with the *C564-GAL4* driver line and the flies were infected with *E. cloacae* to induce the production of AMPs via the Imd pathway. The transcript levels of several Imd-pathway mediated AMPs were analyzed at different time points after infection by qRT-PCR, but no clear effects could be observed (Figure 5C-H in II). However, with the exception of *Drosocin*, *edin* RNAi caused a minor decrease in the transcript levels of the tested AMPs at the 4 h time point, but the decrease was statistically significant only in the case of *Cecropin A1* ($p<0.001$, Figure 5D II) and *Attacin B* ($p=0.01$, Figure 5E in II). Surprisingly, the trend seemed to be the opposite at the 24 h time point, *edin* knock down causing a slight increase in the levels of AMPs. Overexpressing *edin* had likewise no or little effect on the production of AMPs. The levels of *Attacin B*, *Attacin A* and *Drosocin* appeared to increase at the 8 h time point due to the overexpression of *edin*, but the effect was statistically significant only with *Drosocin* ($p<0.05$) (Figure 5E-F and 5H in II). Together, these results show that Edin does not have an important role in the regulation of the immune signaling pathways either *in vitro* or *in vivo*.

5.2.3 Edin in the resistance against bacterial infections (II)

Although Edin did not to have antimicrobial properties or a modulatory role in immune signaling, we further studied the importance of Edin in the immune response of *Drosophila in vivo* by carrying out bacterial infection experiments with *edin*^{14289} RNAi flies. In the experiments, the expression of the RNAi construct was driven in the fat body and some other organs with the *C564-GAL4* driver. The week-old offspring were then infected with *E. cloacae*, *E. faecalis* or *L. monocytogenes*. *E. cloacae* and *E. faecalis* are Gram-negative and Gram-positive bacteria, respectively, whereas *L. monocytogenes* is a Gram-positive bacterium that has a DAP-type peptidoglycan. *L. monocytogenes* was selected for these infection experiments because Edin has been reported to be involved in the pathogenesis of a *Listeria* infection (Gordon et al., 2008). The *edin*^{14289} RNAi flies crossed with the *C564-GAL4* driver had a lowered survival rate compared to *edin*^{14289} x *w*^{1118} control flies, but they survived better than the driver control (*C564-GAL4* x *w*^{1118}), and therefore the reduction could not be considered significant (Figure 8A in II). However, a statistically significant decrease in the survival was observed when *edin* was knocked down in the context of an *E. faecalis* infection (Figure 8B in II). Although Edin has been reported to be important in the resistance against *L. monocytogenes*, in our hands, driving *edin* RNAi with *C564-GAL4* did not significantly lower the survival rate of the flies (Figure 8C in II). However, a similar trend was observed as reported by Gordon et al. (Gordon et al., 2008). These results indicate that Edin might have a role in the resistance against bacterial infections, at least in the case of *E. faecalis*, but further studies are required to elucidate the mechanistic role of Edin in bacterial infection.

5.3 Edin is induced by a wasp infection in *Drosophila* larvae (III)

In addition to microbes, fruit flies are infected by parasitic wasps in their natural environments, and this causes changes in the expression of *Drosophila* genes (Schlenke et al., 2007; Wertheim et al., 2005). Because Edin did not seem to have an important role in the context of a bacterial infection, we wanted to know whether the expression of *edin* could also be induced by a wasp infection, as it is a natural parasite of the fruit fly. Again, we used the wild-type *Canton S* line and infected 1st or early 2nd instar larvae with the *Leptopilina boulardi* strain G486, extracted total RNAs from whole larvae 3 h post infection, and analyzed the transcript levels of *edin* with qRT-PCR. Our data showed that the wasp infection resulted in a 6.7-fold induction

in the expression levels of *edin* in *Canton S* larvae, demonstrating that *edin* can be induced by a wasp infection (Figure 11A). We then further analyzed the induction pattern of *edin* during a wasp infection and extracted total RNAs from the fat bodies of wasp infected *Canton S* larvae. The qRT-PCR results showed that the induction level was higher in the fat bodies than in whole larvae (~80-fold induction), indicating that the fat body was most likely the predominant organ for *edin* expression (Figure 11B). Our results show that in addition to a bacterial infection, *edin* is induced by a wasp infection, and that the fat body seems to be the major organ for its expression.

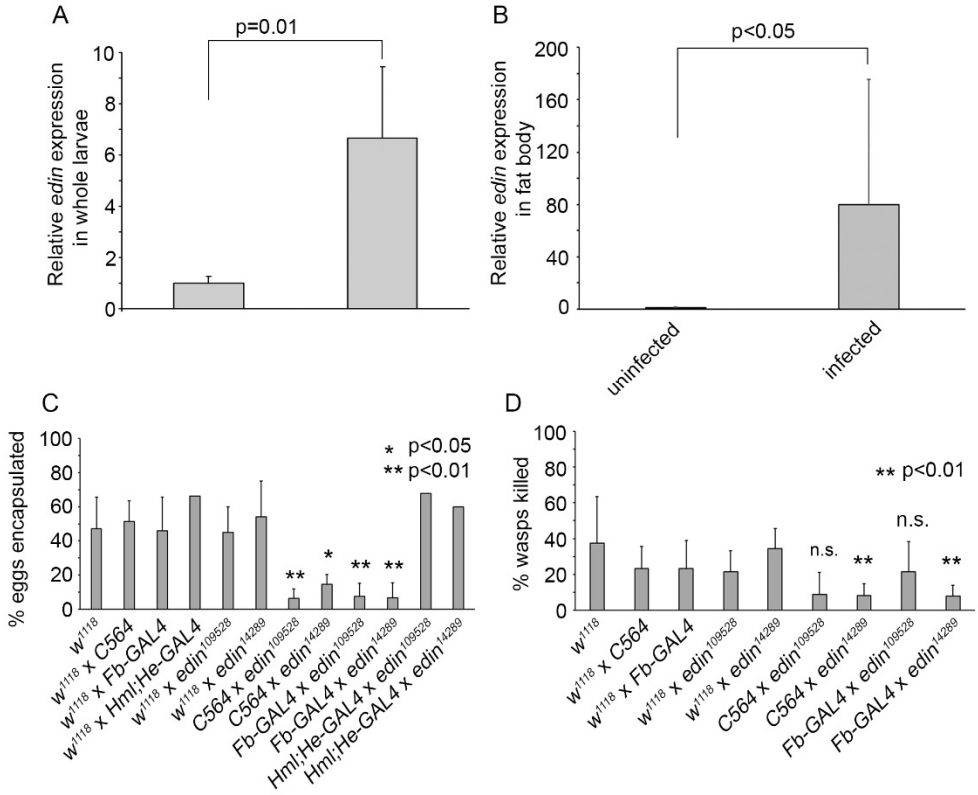

Figure 11. The expression of *edin* is induced in response to a wasp infection and is required for a successful encapsulation response. (A-B) The expression of *edin* is upregulated upon a wasp infection. (C-D) The expression of *edin* in the fat body, but not in hemocytes, is important in the defense against wasp parasitism. (Modified from original publication III)

5.3.1 Edin expression in the fat body is important for the encapsulation response (III)

The major defense mechanism against wasp parasitism in *Drosophila* is the encapsulation of the invading wasp egg by hemocytes. In a successful encapsulation reaction, the wasp egg becomes surrounded by a melanized capsule and is killed inside it. The encapsulation response requires the concerted action of all of the different hemocyte types. Especially the lamellocytes are considered as the hallmark cells of the encapsulation response, as their formation is induced by a wasp infection. To study the functional role of Edin in the encapsulation response, we infected fruit fly larvae with wasps and monitored the formation of a melanotic capsule around the wasp eggs 27-29 h after wasp infection, at which point, the capsule is already melanized, but the wasp larva has not yet hatched from the egg. In our control larvae, 45-66% of the infected larvae contained a melanized capsule, which was then set as the normal encapsulation rate for our experiments (six left-most columns in Figure 11C).

Because hemocytes are important players in the encapsulation response, we first knocked down *edin* in hemocytes using the *Hml$^{\Delta}$;He-GAL4* driver. Both *edin* RNAi lines (*edin^{109528}* and *edin^{14289}*) were analyzed and were found to have encapsulation rates similar to those of the controls (68% and 60%), indicating that the expression of *edin* in hemocytes is not necessary for the encapsulation response (Figure 11C). This finding led us to hypothesize that the expression of *edin* might be needed in the fat body. This idea was further supported by the qRT-PCR data that showed that *edin* was highly induced in the fat body upon a wasp infection (Figure 11B). Therefore, we next used the *C564-GAL4* driver to drive the expression of the *edin* RNAi constructs in the fat body as well as in some other tissues (Harrison et al., 1995). This resulted in significantly reduced encapsulation rates of 6% and 14% for *edin^{190528}* and *edin^{14289}*, respectively (Figure 11C). Because the decrease in the encapsulation rate was observed with both RNAi constructs, it was unlikely that the encapsulation phenotype would have been caused by an artefact of the genetic background of the flies or simply by the presence of the RNAi construct.

As is discussed above, the *C564-GAL4* driver is expressed in many tissues including the fat body, and in order to confirm the role of the fat-body dependent expression of *edin* in the encapsulation response, we next used a fat-body specific driver, *Fb-GAL4*. Again, both *edin* RNAi constructs were crossed with the driver line and the encapsulation response was analyzed 27-29 h after the wasp infection. As a result, *edin* knock down with the *Fb-GAL4* driver caused a significant decrease in the

encapsulation rates in both *edin*^{14289} and *edin*^{109528} RNAi larvae (7% and 8%) indicating that Edin is required in the fat body, but not in hemocytes, for a normal encapsulation response upon a wasp infection (Figure 11C).

Even if the fruit fly larva manages to successfully encapsulate a wasp egg, it might not be able to kill the wasp inside the capsule. In order to investigate the role of Edin in the killing of the wasp larva, we repeated the experimental setting of the encapsulation assay, but scored for the presence of dead wasp larvae 48-50 h post wasp infection. The wasp larva was considered dead if an intact melanized wasp egg was present in the fruit fly hemolymph without the presence of a living wasp larva. If the fly larva had not managed to kill the wasp, there was either a living wasp larva with remnants of a melanized capsule or only a living wasp larva present in the hemocoel. Knocking down *edin* with the *C564-GAL4* driver led to a decrease in the killing ability of the fruit fly larvae compared to the controls. With the *edin*^{14289} construct, the proportion of dead wasp larvae was reduced to 8%, when in controls the proportion of killed wasps was 22-38% of all infected larvae (Figure 11D). However, although the expression of *edin*^{109528} also led to a decrease in the amount of dead wasp larvae when driven with the *C564-GAL4* driver (9% of wasps killed), this reduction was not however statistically significant (Figure 11D). Additionally, when *edin* was knocked down in the fat body with the *Fb-GAL4* driver, only the *edin*^{14289} RNAi construct produced a reduction in the amount of killed wasp larvae (8% of wasps killed), whereas the *edin*^{109528} construct did not affect the killing ability of the fruit fly larvae (21% killed wasps) (Figure 11D). Nevertheless, together these results show that the expression of *edin* in the fat body is required for a normal encapsulation response against wasps.

5.3.2 The role of Edin in the regulation of hemocyte activation upon wasp infection (III)

Drosophila hemocytes, and especially lamellocytes, are key players in the encapsulation response of wasp eggs. To study whether *edin* played a role in the formation of lamellocytes, we carried out a flow-cytometry based assay to quantify the amount of hemocytes in wasp-infected *edin* RNAi larvae using the *eaterGFP* and *msnCherry* reporter lines. With the reporter lines, plasmatocytes express mostly *eaterGFP* (green), whereas lamellocytes express *msnCherry* (red) and can be effectively observed only in the infected larvae (Figure 3A-B' in III). Again, the expression of *edin* was knocked down in the fat body using the *Fb-GAL4* driver, and the hemocytes from

the infected larvae were collected and analyzed 27-29 h after the wasp infection. Our flow cytometry data showed that *edin* RNAi larvae had a normal amount of lamellocytes (Figure 12A) and a normal distribution of different hemocyte populations (Figure 12B) when compared with controls, indicating that the impaired encapsulation response was not due to the loss of lamellocytes in the *edin* RNAi larvae. In the controls, however, the number of plasmatocytes increased significantly in the infected larvae compared to the uninfected larvae, but this effect was not observed in the wasp-infected larvae, where *edin* had been knocked down in the fat body (Figure 12A). This result supports the idea that the amount of circulating hemocytes does not increase when *edin* is knocked down in the fat body, most likely because of an insufficient release of hemocytes from the sessile compartment. In fact, when the larvae were visualized under a fluorescence microscope, the banded pattern of sessile hemocytes was lost upon infection in the control larvae, but not in the *edin* RNAi larvae suggesting that Edin production in the fat body was necessary for the activation of the sessile hemocytes upon a wasp infection (Figure 12C-D'; Figure 4 in III). These results suggest that the production of Edin in the fat body is required for the efficient release of sessile hemocytes into the circulation and for a normal encapsulation response.

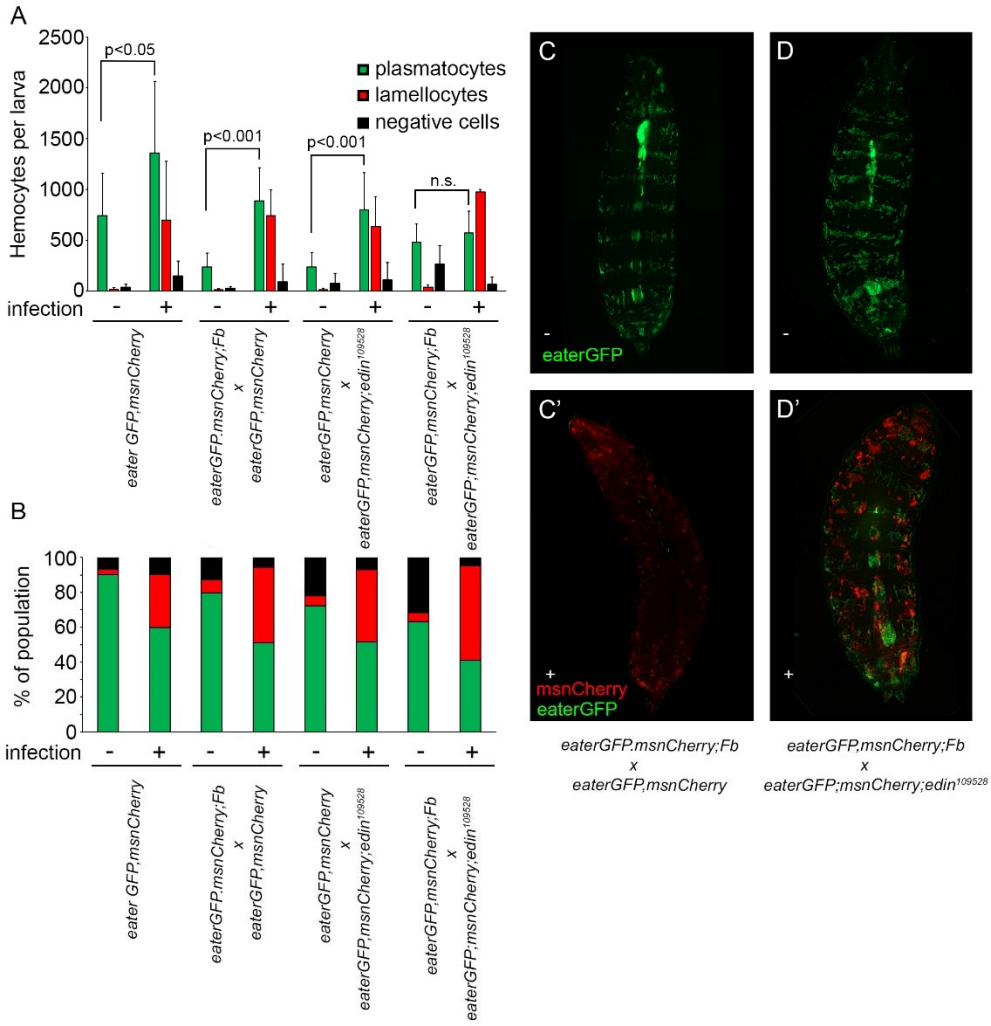

Figure 12. Production of Edin in the fat body is needed for the activation of the sessile hemocyte compartment upon a wasp infection. (A) The number of hemocytes per larva was quantified using flow cytometry and *eaterGFP* (green) and *msnCherry* (red) reporter lines 27-29 h after a wasp infection. Green bars represent plasmatocytes, red bars lamellocytes and black bars non-fluorescent cells. Data were collected 27-29 h post-infection. (B) Proportion of different hemocyte types based on Figure 12A. (C-D') Live imaging of *Drosophila* 3rd instar larvae of the indicated phenotype. (C-D) Uninfected larvae showing the characteristic striped pattern of sessile hemocytes. (C'-D') Infected larvae 27-29 h after wasp infection. A wasp infection triggers the formation of lamellocytes and the release of the sessile hemocytes in the control but not in the *edin* RNAi larvae (D'). - = no infection, + = infection, green = plasmatocytes, red= lamellocytes. (Modified from original publication III)

6 Discussion

6.1 *Drosophila* as a model organism for studying innate immunity

The fruit fly, *Drosophila melanogaster*, has been used in the laboratory since the late 19th century, and is nowadays the model organism of choice for countless scientists. The use of the fruit fly as a model for studying innate immunity did not truly start until the 1970's, when Boman and colleagues began to analyze the inducible humoral immune response in the fruit fly (Boman et al., 1972). After this paper, the work on the humoral aspects of the *Drosophila* immune response started, leading to the discovery of several antimicrobial peptides, the *Drosophila* NF-κB transcription factors, and the Imd and Toll pathways, which were discovered to be central for the regulation of the production of the AMPs (reviewed in Imler, 2014). Alongside the vast amount of research done on the humoral side of the fly's immune response, the interest in the cellular mechanisms of *Drosophila* innate immunity has grown, even though the first studies on cellular immunity were conducted already in the 1970's, concentrating on the mechanisms of encapsulation and melanization in response to a wasp infection.

During the last decades, the fruit fly has proven to be a valid model for studying innate immunity. A major advantage of using *Drosophila* is the low genetic redundancy of the fly genome, and the fact that the fruit fly's immunity relies solely on innate defenses, making it easier to analyze results and to identify interesting phenotypes compared to vertebrate or mammalian models. At a first glance, humans and fruit flies appear to be miles apart in terms of their physiology, but the basic mechanisms of innate immunity are well conserved between the two species. An example of the evolutionary conservation is the role of the fly Toll receptor and the mammalian TLRs in immunity, the latter having been discovered shortly after the significance of the fly Toll for the antifungal defense was deciphered (Lemaitre et al., 1996; Medzhitov et al., 1997; Rosetto et al., 1995). The similarities between the immune systems do not, however, end at the signaling pathways. Like fruit flies, humans are also known to produce AMPs, probably most notably Defensins, in response to an infection (reviewed in Hoffmann et al., 1999).

Apart from the humoral mechanisms, the cellular aspects of *Drosophila* immunity are also well conserved. Although not all *Drosophila* hemocyte types have a counterpart in humans, the fruit fly has professional phagocytes that make it possible to study the mechanisms of phagocytosis, and lamellocytes and crystal cells that take part in the encapsulation process, which could be used to model granuloma formation in mammals. Furthermore, *Drosophila* hemocytes have proven to be a valuable resource in modeling tumorigenesis and tumor-related inflammation (reviewed in Wang et al., 2014). The modeling of cancer and other human diseases is made possible by the fact that 77% of the human genes known to be associated with disease have homologs in *Drosophila* (Veraksa et al., 2000). However, when using the fruit fly, or other model organisms, it must be remembered that it is rarely possible to directly extrapolate the results to humans.

In addition to being a low-maintenance laboratory organism, its small size, fast life cycle and ethical simplicity speak in favour of using the fruit fly as a model organism. The fruit fly offers a large selection of both *in vitro* and *in vivo* research tools that are also applicable for immunological research. For instance, several *Drosophila* cell lines are available, such as the S2 cell line, which is of embryonic origin (Schneider, 1972). The S2 cells are macrophage-like cells that express many immunity-related genes, and are also capable of phagocytosis (Rämet et al., 2001; Rämet et al., 2002b; Samakovlis et al., 1990). The ease of culturing S2 cells has made them a valuable resource for large-scale screening in, for example, microarray studies, and since 2002 RNAi screening methods have also been available (De Gregorio et al., 2001; Irving et al., 2001; Kallio et al., 2005; Kleino et al., 2005; Rämet et al., 2002b; Ulvila et al., 2011a; Valanne et al., 2007; Valanne et al., 2010). The S2 cells readily take up dsRNAs from the culture medium by a scavenger-receptor mediated mechanism leading to the efficient and specific gene silencing by RNAi (Clemens et al., 2000; Ulvila et al., 2006). However, both screening methods have their limitations, as not all genes identified in microarray studies have been discovered in RNAi screens (Kallio et al., 2005; Valanne et al., 2007). Also, the possible off-target effects of dsRNAs may cause false results in the screens, but these can be circumvented with a further step to validate the findings. The genes of interest that were selected for the present study were originally identified in such large-scale screens, the first being a microarray study for genes induced by an *E. coli* infection and the second an RNAi screen for genes affecting phagocytosis in S2 cells (Ulvila et al., 2011a; Valanne et al., 2007). Nevertheless, using *in vitro* models requires careful consideration for many reasons. For instance, the immortalized cell lines might not express all of the genes expressed *in vivo*, and the lack of tissue-to-tissue interaction

might impede the interpretation of the results. Therefore, an *in vivo* validation of an *in vitro* finding is often justified and necessary.

Several *in vivo* methods are relatively freely available and more are being constantly developed thanks to the active nature of the *Drosophila* research field. A very convenient tool, which has been used on a routine basis in this study, is the UAS-GAL4 system that enables the spatio-temporal control of gene expression (Brand and Perrimon, 1993). This is achieved by crossing the flies carrying the tissue-specific transcriptional activator GAL4 with a fly line carrying the transgene or RNAi construct of choice coupled to a UAS-binding site. The F1 progeny then express the construct corresponding to the expression pattern of the GAL4-associated promoter. Nowadays, a large pool of different GAL4 drivers is available making it possible to study the effect of the gene of interest in the cells or tissues of interest without the need to create several transgenic lines with different tissue specificities. However, the choice of the GAL4 driver must be made carefully, as many drivers are expressed in several tissues, whereas others are more tissue specific (for example Goto et al., 2003; Harrison et al., 1995; Schmid et al., 2014; Zettervall et al., 2004). The insertion site of the transgene or the RNAi construct may also cause unwanted off-target effects or lead to false positives and negatives as well as variable expression levels. In the case of publicly available RNAi lines, the effect of the off-target effects has been minimized with the creation of an RNAi library that takes advantage of phiC31-mediated site-specific integration instead of random P-element mediated integration (Dietzl et al., 2007; Ni et al., 2008), but the reality seems to be more complicated as phenotypes not related to the nature of the knock down have been reported to occur (Green et al., 2014). Nevertheless, the combination of the UAS-GAL4 system, the availability of transgenic flies and genome-wide RNAi libraries provide powerful methods for studying gene function by, for example, enabling genome-wide *in vivo* screening in different tissues(Cronin et al., 2009; Kambris et al., 2006; Lesch et al., 2010).

The use of the UAS-GAL4 system also allows for the expression of several constructs in the same fly via selective crossing and thus enabling, for example, epistatic experiments. Though both constructs will then be, of course, expressed in the transcriptional pattern of the same GAL4 driver. An interesting addition to the drosophilist's tool box is the adaptation of the Q system from *Neurospora crassa*, which works similarly to the UAS-GAL4 system (Potter et al., 2010; Potter and Luo, 2011). In the future, a combination of these two systems in one fly could be used to dissect complicated regulatory networks in innate immunity providing an additional level for functional studies.

6.2 14-3-3ζ as an evolutionarily conserved regulator of phagocytosis

The receptors involved in microbial recognition in the fruit fly are relatively well known and intensively studied (Kocks et al., 2005; Kurucz et al., 2007; Philips et al., 2005; Rämet et al., 2001; Rämet et al., 2002b; Stuart et al., 2005; Watson et al., 2005). However, the roles of downstream components involved in the phagocytic cascades are less well studied, although several screens identifying genes involved in phagocytosis have been carried out (Agaisse et al., 2005; Cheng et al., 2005; Derre et al., 2007; Garg and Wu, 2014; Garver et al., 2006; Koo et al., 2008; Pearson et al., 2003; Philips et al., 2005; Rämet et al., 2002b; Stroschein-Stevenson et al., 2006; Stuart et al., 2005). Most likely the mechanism of action of the phagocytic receptors has been easier to determine due to their essential and relatively straight-forward role in the recognition of microbes, whereas many of the downstream components are involved in the remodeling of the actin cytoskeleton, which is known to be essential for the engulfment of microbes (Pielage et al., 2008; Rämet et al., 2001) as well as for the development and homeostasis of the cells. Therefore, many components identified in phagocytosis screens can affect the general viability of the cells, and further studies may prove to be difficult (for example Ulvila et al., 2011a). In addition, many vital functions in the cell are controlled by redundant proteins, possibly explaining why in some cases only mild phenotypes might be observed with genetic manipulations. Nevertheless, redundancy helps to avoid the possible lethal effects caused by the loss of a gene's function.

In original article I, a large-scale RNAi screen was conducted. This led to the identification of three evolutionarily conserved gene products involved in bacterial phagocytosis, namely Abi, cpa and 14-3-3ζ, which are associated with the remodeling of the actin cytoskeleton through the involvement of Actin-related protein 2/3 (Arp2/3). Of the hit genes, only *Abi* and *cpa* had come up in previous phagocytic screens: Abi had been linked with the phagocytosis of *Candida albicans* and cpa with the phagocytosis of *M. fortuitum* and *L. monocytogenes* (Agaisse et al., 2005; Philips et al., 2005; Rämet et al., 2002b; Stroschein-Stevenson et al., 2006). This emphasizes the need for different screening approaches, as different gene products may be identified depending on the experimental set up. 14-3-3ζ on the other hand, had not been previously studied in the context of phagocytosis, but it has been reported to accumulate in phagosome preparations derived from S2 cells (Stuart et al., 2005). In *Drosophila,* two 14-3-3 proteins, 14-3-3ζ and 14-3-3ε, have been identified. 14-3-3 proteins are known to function, for example, as adaptor proteins in the regulation

of the actin cytoskeleton through their interaction with cofilin (Sluchanko and Gusev, 2010). In its active, dephosphorylated form, cofilin binds actin and destabilizes the actin filaments, whereas in its inactive, phosphorylated form, cofilin is unable to bind to actin leading to the stabilization of the actin filaments. 14-3-3 proteins have been reported to interact with both the cofilin kinase LIM and the slingshot family of protein phosphatases (Birkenfeld et al., 2003; Soosairajah et al., 2005), suggesting an essential role for 14-3-3ζ in the regulation of the dynamics of the actin cytoskeleton (Supplementary Figure 3 in I).

With knock-down experiments carried out both *ex vivo* and *in vivo* we were able to show that 14-3-3ζ is an essential component of the phagocytic pathway and that it is required for the resistance against *S. aureus*, where phagocytosis has been reported to play an essential role (Charroux and Royet, 2009; Defaye et al., 2009). Still to obtain reliable results, both *ex vivo* and *in vivo* phagocytosis experiments must be meticulously controlled, because several variables may affect the results. For example, the number of hemocytes in the *Drosophila* larva as well as the phagocytic efficiency of the cells, depend significantly on the developmental stage of the animal (Lanot et al., 2001). Similarly, when studying phagocytosis *in vivo* in adults, the phagocytosing hemocytes are visualized through the pigmented cuticle, which may interfere with the visualization of the fluorescence, but also the size of the body cavity and the amount of phagocytically active hemocytes may vary. Further confirmation for the role of 14-3-3ζ in the phagocytosis of bacteria was obtained by showing that its mouse and zebrafish homologs, Ywhaz and Ywhab, respectively, were also found to be essential for the efficient phagocytosis of bacteria (Ulvila et al., 2011a). In addition, the other member of the *Drosophila* 14-3-3 protein family, 14-3-3ε, has been reported to regulate the production of AMPs by the fat body via the endocytic pathway (Shandala et al., 2011). As phagocytosis is a specialized form of endocytosis, 14-3-3ε might also be involved in phagocytosis similarly to its family member 14-3-3ζ.

By demonstrating the role of 14-3-3ζ in bacterial phagocytosis, our study shows that the fruit fly is a valid model for studying the function of genes acting downstream of phagocytic receptors. Furthermore, the evolutionary conservation of gene products from fly to man makes it feasible to conduct functional studies on phagocytosis in an organism with lower genetic redundancy compared to vertebrate models. Our results also highlight the importance of phagocytosis not only in the recognition of bacteria, but also in the normal host defense of the fruit fly, and, thus confirming similar observations made by Charroux and Royet (2009) and Defaye et al. (2009).

6.3 *Edin* as an immune-inducible gene

Although some genes are constitutively expressed or show at least some baseline expression, the expression of many genes is induced only after an immune challenge demonstrating the importance of the regulation of gene expression in response to an infection (De Gregorio et al., 2001; Irving et al., 2001). The changes in gene expression must be tightly controlled and relatively quick to respond in order for the fruit fly to mount an efficient immune response against the pathogen (for example Boutros et al., 2002; Valanne et al., 2007). The infection-inducible gene *edin*, which was studied in original publications II and III, was identified in a screen for genes induced by *E. coli*. This screen also identified, for example, pirk (Valanne et al., 2007), which was later found out to act as a negative regulator of the Imd pathway (Aggarwal et al., 2008; Kleino et al., 2008; Lhocine et al., 2008). Edin had been previously identified as a novel immune-induced, signal-sequence containing peptide that was secreted into the hemolymph of third instar larvae in response to a bacterial infection (Verleyen et al., 2006). Despite being one the most highly induced genes in the microarray published by Valanne and coworkers (2007), deciphering the significance of Edin for the immune response of the fruit fly proved to be difficult. Besides coming up in the two screens mentioned above, Edin has been identified in several other screens, implying that Edin might play a complex role in the immune defense of the fruit fly. Firstly, Edin has been reported to be differentially expressed in flies with mutations in the gene *mustard*, which is involved in the modulation of the Imd signaling pathway (Wang et al., 2012). Secondly, the study conducted by Short and Lazzaro with unmated and mated female flies also shows that *edin* is induced in response to a bacterial infection (Short and Lazzaro, 2013). Furthermore, Gordon and coworkers, who showed that Edin is required for the defense against an *L. monocytogenes* infection, reported that *edin* is highly induced in both uninfected, and more notably, in infected mutants for WntD (Gordon et al., 2008) that acts as a target for Toll signaling and inhibits the nuclear localization of Dorsal (Gordon et al., 2005). In addition, in original publication III, we show that the expression of *edin* is induced in response to a wasp infection. The induction of *edin* could also be caused by the bacteria transmitted via the wound site during the oviposition of the wasp egg, but this is a highly unlikely scenario, because we were able to show that Edin has an important role in the defense against wasps as it functions as a determinant of the encapsulation response. Surprisingly however, Edin did not come up in the genome-wide expression studies done on wasp-infected flies (Schlenke et al., 2007; Wertheim et al., 2005). Finally, in addition to being directly connected with

immunity, Edin has also been associated with the stress response and amino acid catabolism (Durdevic et al., 2013; Esslinger et al., 2013).

6.3.1 The role of Edin in a bacterial infection

Despite the fact that *edin* has been reported to be upregulated in response to a bacterial infection in several publications (Gordon et al., 2008; Valanne et al., 2007; Verleyen et al., 2006), it has been hard to establish a role for Edin in the defense against bacteria. Although Edin has been linked to the host defense against the DAP-type PGN-containing *L. monocytogenes* (Gordon et al., 2008), we observed only a modest indication for Edin's involvement in a *Listeria* infection. The discrepancy between our study and the study by Gordon and coworkers (2008) could be explained by differences in the genetic background of the flies, the different GAL4 drivers used or by differences in the bacterial dose used in the infection studies. Furthermore, in our hands Edin seemed to have a minor role in the defense against *E. faecalis* (Vanha-aho et al., 2012). The result is somewhat surprising, because *E. faecalis* is known to have a Lys-type PGN, which is an activator of the Toll pathway (Leulier et al., 2003). But in line with the results obtained by Gordon and coworkers (Gordon et al., 2008), we found that the transcription of *edin* is regulated via the Imd pathway (Vanha-aho et al., 2012). In addition, the activation of the Toll pathway was not sufficient to induce the expression of *edin*. Thus, the biological significance of Edin in the bacterial defense and the associated signaling pathways remains a question mark.

A drawback of the experimental set up used to study the role of Edin in bacterial defense, as well as many other infection studies, is the use of a bacterium that is not a natural pathogen of the fruit fly. The unnatural pathogens might elicit a strong immune response in the fruit fly leading to high induction levels in gene expression studies, but some of the upregulated genes might prove to have little actual effect on immunity. The problem with using unnatural pathogens is that they have not coevolved with the host, and might not have evolved efficient attack mechanisms, whereas the host (i.e. the fruit fly) might not have efficient defense mechanisms against the pathogen. Furthermore, fruit flies are most often infected orally through the digestive system or via the trachea in their natural habitat, but many experimental procedures, as the one used in this study, take advantage of the ease of pricking the cuticle with a bacteria-contaminated needle. However, pricking the cuticle with a needle can be used to model the infection caused by pathogens transmitted through

a wounded cuticle, which occurs in nature for example in the event of a wasp infection. Though it is easy and fast to infect a large number of flies with the bacteria-contaminated needle, and the flies recover quickly from the pricking, the amount of bacteria introduced into the body cavity with the needle is difficult to control. This problem can be circumvented by growing the bacteria to a certain optical density, as was done in original publication I, or by using a microinjector to inject a specific volume of bacteria.

To determine the role of Edin in the host defense against bacteria, it could be interesting to carry out infection experiments with naturally occurring pathogens of the fruit fly that have been used in other reports, such as *Pseudomonas entomophila* (Buchon et al., 2009a; Chakrabarti et al., 2012) and *Erwinia carotovora carotovora* (for example in Buchon et al., 2009b). Instead, we used the parasitic wasp, *Leptopilina boulardi*, to study the role of Edin in immunity in a more natural context, which will be discussed in the next chapter.

6.3.2 Edin in the cellular response against parasitic wasps

One of the naturally occuring parasites of the fruit fly is the parasitoid wasp, which elicits a strong immune response in the fruit fly larva requiring the activation and mobilization of hemocytes, the formation of a multilayered capsule around the wasp egg as well as the melanization of the capsule through the activation of the phenoloxidase cascade (reviewed in Carton et al., 2008). Moreover, wasp parasitism induces changes in gene expression in the fruit fly, leading to the upregulation of several genes (Schlenke et al., 2007; Wertheim et al., 2005), including several serine proteases involved in the activation of prophenol oxidases, genes linked to hemocyte development and even some *AMP* genes, though most genes identified in the wasp microarrays differ significantly from the genes induced by a bacterial challenge (Schlenke et al., 2007; Wertheim et al., 2005). The difference in the gene expression reflects the difference in the immune responses required after a microbial challenge and a wasp challenge, the former requiring the release of humoral factors such as AMPs and the latter the proliferation and differentiation of hemocytes. Therefore, it was interesting to observe that Edin, which was highly upregulated after a bacterial infection, seemed to also respond to a wasp infection in whole larvae and even more significantly in the fat body. However, Edin was not detected in the published wasp microarrays, probably due to a low induction level in whole larvae and because the

induction was below the set threshold value (Schlenke et al., 2007; Wertheim et al., 2005).

The main pathways linked with cellular immunity are the Toll, JAK/STAT and JNK pathways (Gao et al., 2009; Krzemien et al., 2007; Minakhina et al., 2011; Schlenke et al., 2007; Schmid et al., 2014; Sorrentino et al., 2002; Wertheim et al., 2005; Williams et al., 2007; Zettervall et al., 2004). Evolutionary studies have also shown that the genes important for the encapsulation response and for the resistance against wasps are genes that have been linked with hemocyte differentiation (Jalvingh et al., 2014; Salazar-Jaramillo et al., 2014). Instead, Imd signaling has not been implicated in cellular immunity and has been shown not to be important for the encapsulation response or for cellular immunity in general (Hedengren et al., 1999). In original publication III, we observed that the expression of *edin* in the fat body is required for a normal encapsulation response and for the activation of hemocytes upon a wasp infection. Based on our experiments, the expression of *edin* does not seem to be mediated via the Toll pathway but via the Imd pathway implying that Imd signaling could be important for the cellular immune response after all. Still, it is also possible that Edin is regulated differentially in the context of a bacterial challenge and a wasp infection. Nevertheless, further experiments are required in order to elucidate the regulatory mechanisms of Edin in the immune defense against wasps.

Based on the major effect of *edin* knock down on the encapsulation response, it would have been tempting to hypothesize that Edin is required for the differentiation of lamellocytes, as they are known to be an essential component of the capsule. This hypothesis could have been further supported by the finding of Gordon and coworkers (2008), who show that the expression level of *edin* is highly elevated in *wntD* mutants, since Wnt signaling has been reported to regulate lamellocyte formation (Zettervall et al., 2004). However, according to our results, knock down of *edin* does not affect lamellocyte formation. Instead, the number of plasmatocytes does not increase in *edin* RNAi larvae as it does in the controls after a wasp infection. Reports have shown that increased hemocyte numbers (plasmatocytes/lamellocytes) have been associated with an increased resistance against wasp parasitism (Kacsoh and Schlenke, 2012; Kraaijeveld et al., 2001; Moreau et al., 2005; Prevost and Eslin, 1998), possibly explaining the lowered resistance against an *L. boulardi* infection as *edin* RNAi larvae have fewer circulating plasmatocytes. Based on the low number of circulating plasmatocytes after a wasp infection, Edin seems to regulate the release of sessile hemocytes into the body cavity. Our study provides the first evidence for the importance of the amount of circulating hemocytes in capsule formation. The

mechanisms behind this phenomenon still remain unclear, but Edin might act as a cytokine-like molecule that is produced in the fat body in response to a wasp infection thus triggering changes in the adhesive properties of the sessile hemocytes and advancing their mobilization. In general, the mechanisms underlying the encapsulation response are still not well understood and deciphering the function of Edin may in the future help to improve our understanding of the host-parasite system.

The wasp is a natural enemy of the fruit fly, and the wasp-fruit fly system thus provides a valid infection model for studying the mechanisms of innate immunity. Because parasitism triggers a vigorous cellular immune response, the model can be used to study the processes of blood cell activation, proliferation and differentiation. Although all of the *Drosophila* hemocyte types do not have a counterpart in humans, some of the signaling pathways controlling blood cell development and the mechanisms of hematopoiesis are evolutionarily conserved. Because the encapsulation response is also said to resemble the formation of a granuloma by macrophages and other blood cells in humans, it may also prove to be a valid model for studying the pathogenesis caused by intracellular bacteria, such as *Mycobacterium tuberculosis*.

6.4 Cross-talk between cellular and humoral immunity

The fruit fly has a rather simple, yet elegant immune system, and the fly can efficiently defend itself against a large variety of pathogens. The fat body of the fruit fly is of pivotal importance in the humoral arm of the immune defense in the production and secretion of antimicrobial peptides that are active against a wide variety of microbes, whereas the *Drosophila* hemocytes are responsible for the cellular arm of the immune defense in the form of microbial phagocytosis and the encapsulation of larger objects. In reality, the humoral and cellular defense mechanisms are closely integrated and form a complex network of interactions. Only relatively recently, reports have started to come out clarifying the processes and molecules involved in the tissue-to-tissue signaling during an immune challenge. Also the results presented in the original publications of this thesis add further knowledge to the understanding of the interaction between the cellular and humoral aspects of *Drosophila* immunity.

The first reports showing evidence for the cross-talk between hemocytes and the fat body came in the early 2000's when Agaisse and coworkers described the role of

Upd3 in the activation of JAK/STAT signaling in the fat body (Agaisse et al., 2003). They showed that hemocytes produce the cytokine-like Upd3, which signals the activation of the JAK/STAT pathway in the fat body, but the signaling events leading to the production of Upd3 remained unclear (Agaisse et al., 2003). Irving et al. have also reported that Spätzle is upregulated in the hemocytes and not in the fat body, probably implying that Spätzle produced by the hemocytes, activates Toll signaling in the fat body (Irving et al., 2005). This finding was later corroborated by the discovery that the expression of *spätzle* in the hemocytes is required for normal AMP production in the fat body (Shia et al., 2009), but reports have also shown that AMPs are produced normally even in the absence of hemocytes (Charroux and Royet, 2009; Defaye et al., 2009).

Other molecules produced by hemocytes have also been associated with a normal fat-body mediated AMP response. These include nitric oxide and Psidin that promote AMP expression in the fat body (Brennan et al., 2007; Dijkers and O'Farrell, 2007; Foley and O'Farrell, 2003). In addition to being linked with AMP production, Psidin was shown to be required for the degradation of phagocytosed bacteria, highlighting the role of phagocytes in the host defense of *Drosophila* and in the activation of the humoral immunity (Brennan et al., 2007). Even though, based on the results of original publication I, knocking down *14-3-3ζ* in hemocytes did not affect the production of AMPs, the expression of *14-3-3ζ* was required for a normal host defense against bacteria showing that an efficient immune response requires the activity of both aspects of the innate immunity (Ulvila et al., 2011a). Other reports have also proved the importance of phagocytosis for a normal functioning immune system (Charroux and Royet, 2009; Defaye et al., 2009; Kocks et al., 2005).

Based on the reports discussed above, the hemocyte-to-fatbody interaction is active in the immune system of the fruit fly. Only very recently, the first publications describing fatbody-to-hemocyte signaling were published. Parisi et al. showed that Eiger and Spätzle produced by the hemocytes are important in activating the Toll pathway in the fat body, and the resulting decrease in the size of blood cell tumors and tumor cell death (Parisi et al., 2014). Another paper also described the importance of fat-body mediated Toll signaling in the control of hemocyte activation (Schmid et al., 2014). However, Toll signaling in the fat body did not have a significant role in the defense against a wasp infection (Schmid et al., 2014). In original publication III, we provide further evidence showing that an efficient immune response requires a tissue-to-tissue interaction from the fat body to the hemocytes. In the future, it would be interesting to decipher how different tissues

contribute to the systemic immune response and how the cellular and humoral immune mechanisms are coordinated.

7 Summary and Conclusions

The last couple of decades have proven that, due to its efficient and evolutionarily well-conserved immune responses and lack of an adaptive immune defense, the fruit fly, *Drosophila melanogaster*, is an excellent model for deciphering the mechanisms of innate immunity. Furthermore, the vast array of genetic tools available for both *in vitro* and *in vivo* research, which are also applicable to immunological studies, have made the fruit fly a preferred model organism compared to many others.

In this present study, both *in vitro* and *in vivo* tools were used to study the function of selected hit genes that had been previously identified in immunity-related large-scale *in vitro* screens. Especially, tissue-specific knock downs using RNA interference were carried out to study the importance of the selected genes for the immune response of the fruit fly. Our findings include the characterization of the cofilin regulator 14-3-3ζ as an evolutionarily conserved protein, which is required in the phagocytosis of bacteria and which most likely functions in the regulation of the actin cytoskeleton. 14-3-3ζ is also required for the resistance against a microbial infection, providing further evidence for the importance of phagocytosis in the host defense of *Drosophila*. We also show a minor role for the infection-inducible peptide Edin in the response against a bacterial infection, but more importantly, Edin is shown to be needed in the defense against wasp parasitism. Upon a wasp infection Edin, which is produced in the fat body, acts non-cell autonomously regulating the release of sessile hemocytes into the circulation resulting in an efficient encapsulation reaction. In our study, we provide the first quantitative evidence for the significance of the numbers of circulating plasmatocytes for a successful encapsulation response. Our results also provide further evidence for the cross-talk between the humoral and cellular arms of the immune defense, particularly in the fat body to hemocyte signaling.

Taken together, our results show that the immune responses of the fruit fly are based on a complex system, which requires efficient cooperation between both cellular and humoral factors, and cannot be explained as the function of either one of these two arms of the immune defense independently. Instead of focusing on single signaling pathways and molecules, it would be interesting to shift the focus onto how the immune system acts as a whole and how the humoral and cellular

mechanisms of immunity are intertwined and regulated, both spatially and temporally. Due to its small compact genome and low genetic redundancy, the fruit fly will continue to be a valuable tool in solving the mysteries of the immune system, and for providing further insight into the intricate regulatory system that controls the elaborate immune system of humans.

8 Acknowledgements

This PhD thesis was carried out in the Experimental Immunology Research Group at BioMediTech, University of Tampere. I wish to thank the director of BioMediTech, Dr. Hannu Hanhijärvi, for the excellent facilities and a friendly working environment.

I would like express my gratitude to my supervisor Prof. Mika Rämet for taking me into his research group already at the beginning of my undergraduate studies and for giving me the opportunity to do both my master's thesis and my PhD thesis under his supervision. I wish to thank him for making it possible for me to participate in many interesting projects and to learn a countless number of both *in vitro* and *in vivo* techniques. It has truly been a privilege to work in Mika's lab, and I thank him for all the guidance, advice and the time he has given me during the years.

I would like to thank the external reviewers of my thesis, Prof. István Andó and Docent Maria Vartiainen, for their valuable comments and kind words. I would also like to thank Prof. Ulrich Theopold for giving me the honor of being my opponent.

I wish to thank my thesis committee members Prof. Heikki Hyöty and Prof. Dan Hultmark for their advice and encouraging comments about my projects. I would also like to thank Prof. Hultmark for his collaboration and for all of his *Drosophila* expertise he has shared with us during the years.

A big thank you to all of the past and present members of the Experimental Immunology lab, you have made the years in the lab really enjoyable. Particularly, I would like to thank Dr. Susanna Valanne, Dr. Anni Kleino, Dr. Johanna Ulvila, Dr. Henna Myllymäki, Dr. Jenni Kallio, Docent Mataleena Parikka, Kaisa Oksanen, Meri Kaustio, Sanna Harjula, Anni Saralahti, Mirva Järvelä-Stölting, Annemari Uusimäki, Matilda Martikainen, Jenna Ilomäki, Leena Mäkinen and Hannaleena Piippo for all of their support and help with the practical as well as the mental side of my PhD project and especially for all the laughs and nice moments we have shared in the lab and also outside of it. A big thank you to Tuula Myllymäki for her practical help with cooking all the high-quality fly food.

Many thanks also to the wonderful colleagues at BioMediTech, and especially to our neighbors in Prof. Hultmark's Genetic Immunology Research Group. Particularly, I wish to thank Ines Anderl and Dr. Laura Vesala, for sharing their

scientific tools and methods, for their massive efforts with the second Edin paper and also for the great company and for the occational visits to the best (and newest) restaurants in Tampere. I would also like to thank the Umeå section of the group for the nice journal clubs, scientific discussions we have had on many Wednesday mornings via Skype during the years and also for the nice *Drosophila* retreats.

I wish to thank all the co-authors both in Finland and abroad, without whom none of this would have been possible.

Next, I would like to thank my friends outside the lab, who are an important part of my life. My partner in brunches and cakes, Elisa Kaaja, is acknowledged for all the memorable moments we have had and for being such a lovely person to be around with. I wish to thank Auri Kohola for all the baking, eating, traveling and TV nights we have shared together, and also for being the active part in our friendship when I have been "too busy" to keep in touch. I would like to thank Heidi Huovinen for her unconditional friendship and support during the countless years we have known each other. I wish to thank Dr. Kirsti Laurila for all the talks we have had about lab work and life in general as well as for the nice cooking sessions we have had (and maybe also for the irregular shop 'til you drop sessions somewhere abroad). A special place in my heart belongs to my friends at the Tampere Rugby Club, especially to the lovely TRC ladies. Thank you for lightening up my days on and off the pitch for the past years and for always taking an interest in my (weird) science projects. Many thanks to Hanna Visuri, Sonja Kivelä, Maija Kumpula, Alisa Myllynpää, Laura Malmi, Kati Miettinen, Ulla Korpi-Tassi, Heidi Ovaska and all the other rugby sisters who I failed to mention, and especially to Miia Joronen and Riina Mylläri for the cake and candy deliveries and the supporting messages during the writing process of this thesis.

I wish to express my deepest gratitude to my family. First, I would like to thank my wonderful parents, Ulla-Maija and Pentti Vanha-aho, for all of their love, care and encouragement and especially for always believing in me. I would like to thank my big brother Jani Vanha-aho and my big sister Hanna-Kaisa Desavelle for being the best brother and sister one could imagine. I also wish to thank my brother-in-law Jérôme Desavelle for introducing our family to the art of French champagne and cheese, and my lovely nephews Axel and André Desavelle for bringing so much joy to all of our lives. I also wish to thank Kirsti and Pekka Anttila for their warmth and kindness during the years. Last but not least, I want to thank my dearest Matti for his unconditional love and support during the many years we have spent together and for the many years to come.

This work was financially supported by the Tampere Graduate Program in Biotechnology and Biomedicine and the Orion-Farmos Research Foundation.

Tampere, January 2015

Leena-Maija Vanha-aho

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10 Original Communications

JLB

Article

Cofilin regulator 14-3-3ζ is an evolutionarily conserved protein required for phagocytosis and microbial resistance

Johanna Ulvila,[*,†] *Leena-Maija Vanha-aho,*[†] *Anni Kleino,*[†,‡] *Mari Vähä-Mäkilä,*[*] *Milka Vuoksio,*[†] *Sinikka Eskelinen,*[§] *Dan Hultmark,*[†,‡] *Christine Kocks,*[||] *Mikko Hallman,*[*] *Mataleena Parikka,*[†] *and Mika Rämet*[†,¶,1]

Departments of *Pediatrics and [§]Pathology, Biocenter Oulu, University of Oulu, Finland; [†]Institute of Biomedical Technology and [¶]Department of Pediatrics, University of Tampere, Finland; [‡]Department of Molecular Biology, Umeå University, Umeå, Sweden; and [||]Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts, USA

RECEIVED APRIL 8, 2010; REVISED NOVEMBER 24, 2010; ACCEPTED NOVEMBER 29, 2010. DOI: 10.1189/jlb.0410195

ABSTRACT

Phagocytosis is an ancient cellular process that plays an important role in host defense. In *Drosophila melanogaster* phagocytic, macrophage-like hemocytes recognize and ingest microbes. We performed an RNAi-based in vitro screen in the *Drosophila* hemocyte cell line S2 and identified Abi, cpa, cofilin regulator 14-3-3ζ, tlk, CG2765, and CG15609 as mediators of bacterial phagocytosis. Of these identified genes, *14-3-3ζ* had an evolutionarily conserved role in phagocytosis: bacterial phagocytosis was compromised when *14-3-3ζ* was targeted with RNAi in primary *Drosophila* hemocytes and when the orthologous genes *Ywhab* and *Ywhaz* were silenced in zebrafish and mouse RAW 264.7 cells, respectively. In *Drosophila* and zebrafish infection models, 14-3-3ζ was required for resistance against *Staphylococcus aureus*. We conclude that *14-3-3ζ* is essential for phagocytosis and microbial resistance in insects and vertebrates. *J. Leukoc. Biol.* 89: 649–659; 2011.

Introduction

Phagocytosis is an important, evolutionarily conserved innate defense mechanism originating from nutritional uptake in unicellular organisms [1]. In *Drosophila melanogaster,* resistance against infectious microorganisms relies on innate immune responses [2–4]. Normal microbial resistance requires the production of potent antimicrobial peptides upon microbial challenge as well as the recognition and subsequent phagocytosis of bacteria [5–7].

In *Drosophila*, circulating blood cells, plasmatocytes, mediate the phagocytosis of microorganisms and apoptotic cells [8]. Phagocytosis is initiated by the recognition of the particle to be ingested, followed by remodeling of the cytoskeleton leading to the engulfment and destruction of the particle. To date, several recognition molecules with varying specificities toward different pathogens have been characterized. Among these receptors are scavenger receptor class C, type I, CD36 family scavenger receptors Peste and Croquemort, peptidoglycan recognition protein-LC, NIM repeat-containing proteins Eater, Nim1C, and Draper, and Ig superfamily protein Down syndrome cell adhesion molecule, capable of extensive splice variation [5, 9–15].

Drosophila has become a powerful model for investigating the basic mechanisms of phagocytosis. Plasmatocyte-like S2 cells recognize and engulf bacteria in a manner comparable with that of plasmatocytes [9]. Importantly, large-scale, high-throughput RNAi screens can be easily carried out in this cell culture system [10]. To date, several RNAi screens identifying components required for the internalization and/or phagocytosis of various microorganisms have been published [10, 11, 16, 17]. Identified gene products include Arps and mediators of vesicle trafficking and endocytosis. Although numerous gene products have been proposed to participate in the complex process of bacterial phagocytosis, details of the roles and mechanisms of action of these proteins, as well as their in vivo significance for the immune response in *Drosophila* and higher organisms, remain to be assessed and confirmed.

Abbreviations: Abi=Abelson interacting protein, Arp=actin-related protein, ATCC=American Type Culture Collection, BH=brain heart, Capza1=capping protein (actin filament) muscle Z-line, α 1, cpa=capping protein α, dpf=days post-fertilization, Ehbp1=EGFR pathway substrate 15 homology domain-binding protein 1, EHD=EGFR pathway substrate 15 homology domain protein, Hml=hemolectin, Imd=immune deficiency, LIM=Lin-11/Isl-1/Mec-3, MFI=mean fluorescence intensity, Mid1ip1=midline 1-interacting G12-like protein, MO=morpholino, qPCR=quantitative PCR, RNAi=RNA interference, SCAR=suppressor of cAMPR mutation, siRNA=small interfering RNA, SSH=slingshot, tlk=tousled-like kinase, VDRC=Vienna Drosophila RNAi Center, WASp=Wiscott-Aldrich syndrome protein, WAVE=Wiskott Aldrich verpolin homologous protein complex, WPI=World Precision Instruments, Ywhab=tyrosine 3-mono-oxygenase/tryptophan 5-mono-oxygenase activation protein, β polypeptide, Ywhaz=tyrosine 3-mono-oxygenase/tryptophan 5-mono-oxygenase activation protein, ζ polypeptide

The online version of this paper, found at www.jleukbio.org, includes supplemental information.

1. Correspondence: Institute of Medical Technology, 33014 University of Tampere, Finland. E-mail: mika.ramet@uta.fi

To understand the basic mechanisms of phagocytic uptake of bacteria, we carried out an RNAi screen to identify gene products that affect the phagocytosis of *Escherichia coli* in *Drosophila* S2 cells. We then analyzed whether our novel candidate genes have evolutionarily conserved roles in microbial phagocytosis by silencing their vertebrate homologs by siRNA treatments. Furthermore, the in vivo relevance of the gene *14-3-3ζ* was studied using *Drosophila* ex vivo and in vivo phagocytosis assays. Additionally, a novel method for the quantification of bacterial phagocytosis in zebrafish larvae in vivo was developed. This novel method was used to determine the role of *14-3-3ζ/Ywhab* in microbial phagocytosis in a model organism possessing the innate and adaptive branches of the immune response. Finally, the importance of the candidate gene for microbial resistance was studied in vivo in *Drosophila* and zebrafish. Our results indicate that the cofilin regulator 14-3-3ζ has an evolutionarily conserved role in phagocytosis and is required for normal microbial resistance in *Drosophila* and zebrafish.

MATERIALS AND METHODS

Fly lines

14-3-3ζ RNAi lines (*17870 R-1* and *17870 R-2)* were obtained from the NIG-Fly Stock Center (National Institute of Genetics, Mishima, Shizuoka, Japan) and *14-3-3ζ* RNAi line #48725 and *Eater* RNAi line (stock #4301) from VDRC [18]. RNAi flies and Hml^{Δ}-*GAL4* flies crossed over w^{1118} were used as controls. *Eater* null flies were generated as described previously [5] using the deficiency lines *Df(3R)Tl-I/TM3, P{ActGFP}JMR2, Ser1* and *Df(3R)D605/TM3, P{ActGFP}JMR2, Ser1*. Flies were kept at 25°C on mashed potato, syrup, yeast, and agar medium.

Cell culture

Schneider S2 cells and mouse RAW 264.7 cells were cultured as described previously [19, 20].

Synthesis of the dsRNAs

A cDNA library, derived from S2 cells, cloned into the pcDNAI plasmid (Invitrogen/Life Technologies, Carlsbad, CA, USA) [19], was used as a source for templates for RNA synthesis essentially as described earlier [10, 21].

Sequencing

For sequencing reactions, the DNA was purified from the PCR reactions with the GENECLEAN Turbo kit (QBiogene/MP Biomedicals, Solon, OH, USA) or from the agarose gels with the QIAquick gel extraction kit (Qiagen, Hilden, Germany). Samples were prepared for sequencing with ABI BigDye terminators (Applied Biosystems/Life Technologies, Carlsbad, CA, USA), essentially as recommended by the manufacturer. Excess dye-labeled terminators and buffers were removed by ethanol/EDTA precipitation, and DNA sequencing was performed with an ABI 3100 automated sequencer (Applied Biosystems/Life Technologies).

siRNA treatments

The following mouse genes were targeted for silencing using commercial siRNAs (Ambion, Austin, TX, USA, and Applied Biosystems/Life Technologies): *Abi1* (RefSeq number NM_007380), *Abi2* (NM_198127), *Capza1* (NM_009797), *Ehbp1* (NM_153078), *Mid1ip1* (3110038L01Rik, NM_026524), *Tlk1* (NM_172664), *Tlk2* (NM_011903), and *Ywhaz* (NM_011740). Genes were selected for silencing based on their homology to genes identified in the *Drosophila* RNAi screen (Basic Local Alignment Search Tool, National Center for Biotechnology Information, Bethesda, MD, USA). In addition, inparanoid analysis [22] suggested that the mouse genes *Abi2, Capza1*, *Ehbp1, Mid1ip1,* and *Ywhaz* are orthologs of the *Drosophila Abi*, *cpa, CG15609, CG2765,* and *14-3-3ζ*, respectively. Silencer negative control siRNAs (#1, #2, and #5; Ambion and Applied Biosystems/Life Technologies) were used as a negative control. The mixture of three siRNAs/gene at a final concentration of 200 nM was double-transfected in the RAW 264.7 cells using Lipofectamine 2000 reagent (Invitrogen/Life Technologies). In brief, cells were plated onto a six-well plate at the density of 5×10^5 cells/well, onto a 12-well plate at the density of 1.5×10^5 cells/well, or onto a 24-well plate at the density of 1.5×10^5 cells/well, depending on the assay. siRNAs were diluted to a 40-μM concentration in Opti-MEM (Invitrogen/Life Technologies) and mixed with Lipofectamine 2000 reagent. The transfection mixture was incubated at room temperature for 20 min, and the growth medium of the cells was replaced with fresh medium (DMEM supplemented with 10% FBS, 10 mM Hepes, and 5% L-glutamine). The cells were incubated with siRNAs for 4 h, followed by the addition of fresh medium. After 24 h, the cells were retransfected following the same protocol. After a double transfection for a total of 48 h, the experiments were performed as described. Transfection efficiency was tested using nonsilencing Alexa fluor 488-labeled siRNA (Qiagen). To exclude nonspecific effects of the transfection protocol, control experiments were carried out using the transfection reagent without siRNAs and vice versa.

MO gene silencing in zebrafish

MOs were designed to target the translation start sites of *Abi2a* (Ensembl transcript ID ENSDART00000081015; 5′-CTTTACATCTCCCTCTCTCTC-CCTC-3′), *Abi2b* (Ensembl transcript ID ENSDART00000081014; 5′-GC-CATCTTCACATTTACTGATGCA-3′), and *Pu.1* (Ensembl transcript ID ENSDART00000036729; 5′-CCTCCATTCTGTACGGATGCAGCAT-3′; GeneTools, Philomath, OR, USA). MOs against *WASp1* (Ensembl transcript ID ENSDART00000039080) (5′-GCCCTTTGCTTTTGCCTTTGCTCAT-3′), and *WASp2* (Ensembl transcript ID ENDART00000026807; 5′-CTTTC-CCCTTCGCGGCTCGCCTCAT-3′) were designed based on Cvejic et al. [23] with minor modifications. *14-3-3ζ/ywhab* (Ensembl transcript ID ENSDART00000025940) was targeted with translation-blocking MO (MO1: 5′-TCTGCACTAGGTCACTCTTGTCCAT-3′) and a splice-blocking MO binding to the splice donor site at the exon 3-intron 3-4 boundary of *14-3-3ζ/ywhab* (MO2: 5′-TCAAATCAAACAAACTCACTTCTCT-3′). A random control oligo (5′-CCT CTT ACC TCA GTT ACA ATT TAT A-3′) was obtained from GeneTools. MOs were resuspended in sterile water, and 1 nl was injected into the yolk sac of one to four cell-stage embryos at concentrations of 125–500 μM.

qPCR

For total RNA isolation, RAW 264.7 cells were cultured on six-well plates (Greiner Bio-One, Frickenhausen, Germany), and siRNA treatments were performed as described above. Twenty-four hours after the second siRNA treatment, cells were scraped directly into TRI Reagent (Sigma-Aldrich, St. Louis, MO, USA), and RNA was isolated according to the manufacturer's instructions. qRT-PCR was performed using the Quantitect SYBR Green RT-PCR kit (Qiagen) and the ABI7000 (Applied Biosystems/Life Technologies) instrument. Results were analyzed with the ABI7000 system SDS software, Version 1.2.3. *GAPDH* was considered as a stabile housekeeping gene and used as an internal control. The primers used for qPCR are available on request.

Bacterial phagocytosis assay of cultured cells

dsRNAs were introduced into *Drosophila* S2 cells by soaking. On a 48-well plate, 200,000 cells/well were treated with 5 μg dsRNA. After 72 h, the ability of the dsRNA-treated cells to phagocytose heat-killed, FITC-labeled *E. coli* or *Staphylococcus aureus* (Molecular Probes, Leiden, Netherlands) was analyzed using flow cytometry [10, 24]. Extracellular fluorescence was quenched by adding 0.25% Trypan blue in PBS, pH 4.85 (EuroClone, Pero, Italy), just prior to analysis.

For the RAW 264.7 cells, 10^5 cells/well were seeded onto 24-well plates for flow cytometry. Twenty-four and 48 h later, cells were siRNA-treated using Lipofectamine 2000 reagent (Invitrogen/Life Technologies)-mediated transfection. Twenty-four hours after the second transfection, 5×10^6 FITC-labeled *E. coli* or *S. aureus* were added to the fresh growth medium, and cells were allowed to internalize fluorescent bacteria for 45 min at +37°C, after which, the phagocytosis reaction was terminated by transferring the samples onto ice. Cells were detached from the growth support by scraping, resuspended in ice-cold PBS, and analyzed by FACSCalibur (BD Biosciences, Franklin Lakes, NJ, USA).

Phagocytosis assay with primary hemocytes

Homozygous *14-3-3ζ* RNAi flies (#48725) were crossed over the hemocyte-specific *Hml$^{\Delta}$-GAL4* driver [25]. *w^{1118}* flies crossed over *14-3-3ζ* RNAi, or driver flies were used as negative controls. *w^{1118}/Hml$^{\Delta}$-GAL4* larvae were used as a reference for normalization. *Eater* RNAi (VDRC stock #4301) flies, crossed over driver flies, were used as a positive control for defective phagocytosis. The larval hemocytes were obtained from the offspring of the crosses. Ex vivo bacterial phagocytosis assays were performed as described [26] with the following modifications. Equal numbers of male and female late-wandering, third instar larvae were disinfected in a 5% sodium hypochlorite solution for 2 min, washed three times in H_2O, and bled into 330 μl ice-cold Schneider's *Drosophila* medium (Sigma-Aldrich). Then, excess medium was removed, 3×10^6 FITC-labeled, heat-killed bacteria were added and centrifuged briefly onto the cells, and the cells were allowed to phagocytose for 10 min at 25°C. Plates were returned to ice, the cells were fixed with 2% glutaraldehyde at room temperature, and extracellular fluorescent particles were quenched with trypan blue solution. Microscopy and imaging were performed using an Olympus IX71 microscope with an F-view soft imaging system and QCapture Pro 6.0 software.

In vivo phagocytosis assay in *Drosophila*

pHrodo-conjugated heat-killed *E. coli* (Invitrogen/Life Technologies) were resuspended in sterile PBS to the final concentration of 4 mg/ml. The suspension was vortexed, sonicated, and pulled 20 times through a 30-G syringe to avoid aggregation of the bioparticles. The suspension (25 nl) was then injected into 1-week-old healthy male flies using a PV839 Pneumatic PicoPump microinjector (WPI, Sarasota, FL, USA). Control flies were injected with 70 nl sterile PBS or 2× concentrated, surfactant-free carboxylate-modified latex beads (diameter 0.3 μm; Invitrogen/Life Technologies) 24 h before injecting with pHrodo-conjugated bacteria. The flies were incubated for 30 min at room temperature, except for temperature controls, which were incubated at +4°C. Thereafter, the flies were anesthetized with FlyNap® (Carolina Biological Supply Co., Burlington, NC, USA) and mounted on agarose. Imaging was performed using a Zeiss Lumar.V12 stereomicroscope with an AxioCam MRm camera and the AxioVision Rel. 4.8 program. Quantification of the images was performed with ImageJ by measuring the area of pixels with gray value ≥115 and their mean gray value within the fly abdomen. The phagocytic index represents the area multiplied by the mean gray value. A statistical analysis was performed by one-way ANOVA, and $P < 0.05$ was considered significant.

In vivo phagocytosis assay in zebrafish

Heat-killed, FITC-labeled *E. coli* (10 mg; Molecular Probes) were suspended in 1 ml sterile PBS. Phenol red was added as a tracer, and 2 nl of the suspension was injected into the blood circulation (cardinal vein) of anesthetized (0.17 mg/ml MS222, Sigma-Aldrich), 2 dpf zebrafish larvae using a PV839 Pneumatic PicoPump microinjector (WPI). For live imaging, the larvae were embedded in 1% low melting-point agarose, and bacterial phagocytosis was visualized using an Olympus BX61WI fluorescence microscope and the CellM software. Live imaging was carried out 1.5 h after bacterial infection. For quantification of the internalized bacteria, the larvae were incubated for 3 h at +28°C to allow phagocytosis of the injected, heat-killed bacteria, killed with an overdose of Tricaine anesthetic, and dissociated using 0.5% trypsin-EDTA (Sigma-Aldrich; +37°C, 45 min). Before dissociation, 10 larvae were pooled together for each experimental group. The dissociated cell suspension was fixed for 5 min with 4% PFA (Sigma-Aldrich) and resuspended in 500 μl PBS for FACS analysis. The fixed samples were stored at +4°C, and FACS analysis was performed later. The fluorescence from extracellular bacteria bound to the cells was quenched by adding 0.25% Trypan blue in PBS, pH 4.85 (EuroClone), 1:1 to the cell suspension just before analysis. The FACS analysis was performed with BD FACSAria™ (BD Biosciences) and Cyflogic software (CyFlo Ltd., Turku, Finland). From the dot plot of a total cell suspension, the most forward-scatter-positive cells were gated, representing 1–2% of the total cell population. The selected gate was shown to include phagocytic myeloid cells by analyzing *pu.1* MO-targeted samples that lack myeloid cells [27]. From the selected cell population, FITC-positive cells were gated, and the background fluorescence of cells from larvae without bacterial injection was excluded. The phagocytic index was calculated by multiplying the percentage of phagocytosing cells with the MFI of these cells.

Drosophila infection assays

14-3-3ζ RNAi flies (*17870 R-1* and *17870 R-2*) were crossed over *Hml$^{\Delta}$-GAL4* driver flies. *w^{1118}* flies crossed over driver, or *14-3-3ζ* RNAi flies were used as negative controls. For *Enterobacter cloacae* infection, *Relish^{E20}* mutant flies were used as a positive control for a defective humoral immune response. The infection assays were performed as described previously [28] with the following modifications. *S. aureus* strain 29213 (ATCC, Manassas, VA, USA) and *E. cloacae* strain β12 were cultured in BH or LB broth at +37°C to an $OD_{600\ nm}$ of 1.7 and 2.0, respectively. The bacterial suspension was pelleted by centrifugation, and a 1:10 dilution in sterile glycerol was prepared. One-week-old healthy flies were pricked with a tungsten needle, previously dipped into the *S. aureus* or *E. cloacae* dilution. A statistical analysis was carried out using Log Rank analysis. $P < 0.05$ was considered significant.

Zebrafish infection assay

S. aureus strain 29213 (ATCC) was grown on BH infusion plates (Fluka, Switzerland) and in BH broth (Fluka). Bacteria were grown to an $OD_{600\ nm}$ of 1.0, pelleted, and resuspended in 0.2 M KCl. Zebrafish larvae (2 dpf) were anesthetized (0.17 mg/ml MS222; Sigma-Aldrich), and 2 nl injection solution containing 1000 cfu *S. aureus* in 0.2 M KCl, pH 7.2, was injected into the blood circulation valley. Phenol red was used as a tracer dye in the solution. After bacterial injections, the larvae were transferred to 24-well plates and kept in separate wells in 1 ml E3-H_2O at +28°C. Each experimental group consisted of 21–24 larvae. The survival of infected larvae was followed for 96 h. A statistical analysis was carried out using Log Rank analysis.

Confocal microscopy

1.5×10^5 RAW 264.7 cells per well were seeded onto round glass coverslips on 12-well plates for microscopy. Twenty-four hours later, cells were transfected with Lipofectamine 2000 reagent (Invitrogen/Life Technologies) as described above. The transfection was repeated the next day. Twenty-four hours after the second transfection, cells were allowed to internalize heat-killed *E. coli* in fresh growth medium for 5 min at +37°C, after which, cells were washed twice with Hank's buffer and thereafter, fixed and stained. The specimens were viewed with an Olympus Fluoview 1000 confocal microscope equipped with diode, argon, and helium-neon 1 lasers, using ×60 oil immersion objective (numerical aperture: 1.35), appropriate filters, and the FV10-ASW software (Olympus, Tokyo, Japan).

Statistical analysis

Unless stated otherwise, a statistical analysis of results was carried out using one-way ANOVA, and $P < 0.05$ was considered significant.

RESULTS

An RNAi screen in *Drosophila* S2 cells to identify genes required for the phagocytosis of heat-killed *E. coli*

Flow cytometry can be used to accurately measure the rate of binding and engulfment of fluorescently labeled bacteria by *Drosophila* S2 cells [9]. We extended our previous screen [9] to assess the effect of 3048 random dsRNA treatments on bacterial phagocytosis. As shown in **Fig. 1A**, the majority of RNAi treatments had little or no effect on the phagocytosis of *E. coli*. One hundred eleven dsRNA treatments increased the engulfment of heat-killed *E. coli* at least twofold compared with untreated controls (Fig. 1A, six far-right columns). Sequencing of the corresponding cDNA templates revealed that most of these treatments targeted genes encoding ribosomal proteins (data not shown). One hundred nine dsRNA treatments representing 50 different genes caused

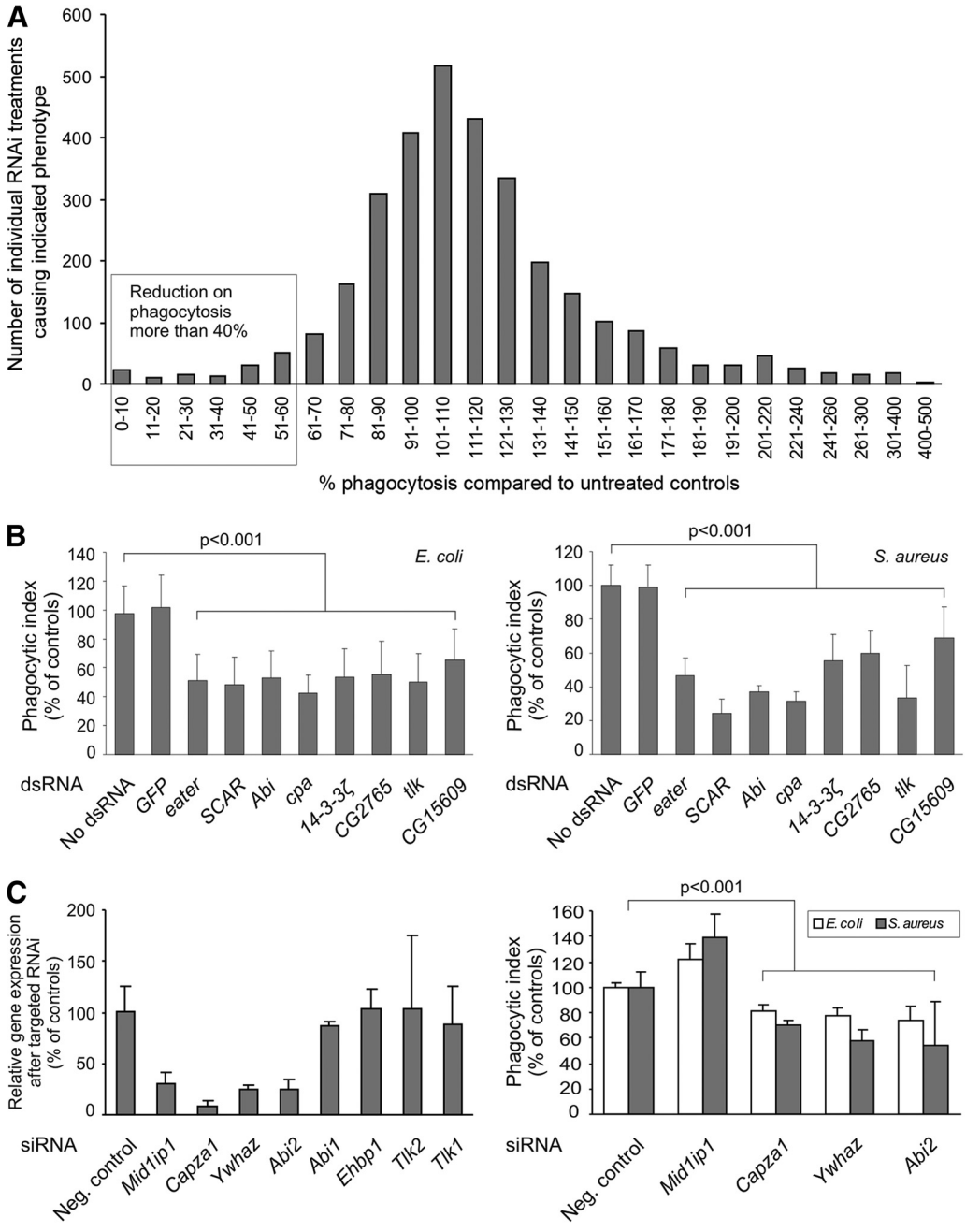

Figure 1. RNAi screen of 3048 dsRNA treatments reveals six gene products required for efficient phagocytosis in *Drosophila* S2 cells. (A) The effects of 3048 random dsRNA treatments on phagocytosis in *Drosophila* S2 cells. The ability of the dsRNA-treated cells to internalize heat-killed, FITC-labeled *E. coli* was analyzed using flow cytometry. Extracellular fluorescence was quenched by adding trypan blue prior to the analysis. dsRNA treatments causing at least a 40% decrease in phagocytosis (six left columns) as compared with untreated control cells were selected for further analysis. (B) Abi, cpa, 14-3-3ζ, tlk, CG2765, and CG15609 play an essential role in bacterial phagocytosis in *Drosophila* S2 cells. dsRNA treatments targeting *Abi, cpa, 14-3-3ζ, tlk, CG2765,* and *CG15609* decrease the phagocytosis of *E. coli* and *S. aureus* in S2 cells. *GFP* dsRNA-treated cells were used as a negative control and *eater* and *SCAR* dsRNA-treated cells as positive controls. The phagocytic index represents the percentage of phagocytosing cells multiplied by the MFI of these cells. Each bar represents the mean ± SD of at least five independent experiments. (C) The mechanisms of phagocytosis are conserved from fruit fly to mammals: orthologs of *Drosophila* Abi, cpa, and 14-3-3ζ participate in the phagocytosis of *E. coli* and *S. aureus* in mouse RAW 264.7 cells. (Left) qRT-PCR analysis of mRNA levels of siRNA-silenced genes in mouse macrophage-like RAW 264.7 cells, which were transfected with pools of three commercial siRNAs to knock down the expression of the mouse homologs of the identified *Drosophila* genes. Total RNA from the siRNA-treated cells was isolated, and the effectiveness of siRNA treatments was analyzed by qRT-PCR. GAPDH was used as an internal control. The results show the level of targeted gene expression in siRNA-treated cells compared with cells transfected with sham siRNAs. Four of the eight siRNA treatments, targeting *Mid1ip1*, *14-3-3ζ/Ywhaz*, *Capza1*, and *Abi2*, effectively silenced the targeted gene. Each bar represents the mean ± SD; $n = 4$. (Right) 14-3-3ζ/Ywhaz, Capza1, and Abi2 are mediators of phagocytosis in RAW 264.7 cells. siRNA-treated cells were incubated with FITC-labeled *E. coli* or *S. aureus*, and the rate of phagocytosis was analyzed using flow cytometry. siRNA treatments targeting *14-3-3ζ/Ywhaz*, *Capza1*, and *Abi2* impaired the ability of RAW 264.7 cells to engulf bacteria compared with cells treated with sham siRNAs. Each bar represents the mean ± SD; $n = 4$.

more than a 40% reduction in phagocytic ability (Fig. 1A, six far-left columns). Most of these dsRNA treatments have been reported to affect the viability of S2 cells, as measured by a luciferase-based assay for ATP levels [29] or a colorimetric assay for *Actin 5C* promoter-driven β-galactosidase activity [30]. The genes affecting phagocytosis as a result of a general effect on viability are listed in Supplemental Table 1. For the remaining genes, we synthesized new dsRNAs and measured the effect of these on the phagocytosis of heat-killed *E. coli* and *S. aureus*. After this double-checking step, our screen identified six genes altogether, the knockdown of which reduced the phagocytosis of *E. coli* and *S. aureus* in *Drosophila* S2 cells: *Abi* (*CG9749*), *cpa* (*CG10540*), cofilin regulator *14-3-3ζ* (*CG17870*), *tlk* (*CG2829*), *CG2765*, and *CG15609* (Fig. 1B). The reduction on phagocytosis varied from 35% to 60% for *E. coli* and from 30% to 75% for *S. aureus*, respectively (Fig. 1B). In all cases, the effect on phagocytosis was similar for *E. coli* and *S. aureus*, representatives of Gram-negative and Gram-positive bacteria, respectively.

14-3-3ζ, Abi, and cpa have evolutionarily conserved roles in phagocytosis

Phagocytosis is an ancient, evolutionarily conserved process, which likely has its origins in the uptake of nutrients by unicellular organisms [1]. Therefore, it is plausible that genes required for the phagocytosis of bacteria in *Drosophila* perform a similar function in mammals. To test this, we knocked down eight homologues altogether of the identified genes in mouse RAW 264.7 cells using pools of commercial siRNAs (Fig. 1C). Four out of eight siRNA treatments (*Abi2*, *Capza1*, *Mid1ip1*, *Ywhaz*) effectively reduced the mRNA levels of the targeted gene, as measured by qRT-PCR (Fig. 1C, left panel). We used flow cytometry to test whether siRNAs for *Abi2*, *Capza1*, *Mid1ip1*, and *Ywhaz* affect the phagocytosis of heat-killed, FITC-labeled bacteria in RAW 264.7 cells. *Mid1ip1* (an ortholog of *Drosophila CG2765*) siRNA-treated cells phagocytosed bacteria normally, if not slightly more efficiently than untreated RAW 264.7 cells (Fig. 1C, right panel). In contrast, *Abi2* (an ortholog of *Drosophila Abi*) RNAi resulted in a 26% and 46% reduction in phagocytosis of *E. coli* and *S. aureus*, respectively. Likewise, phagocytic indexes decreased with *Capza1* (ortholog of *Drosophila cpa*) RNAi by 18% and 30% and with *Ywhaz* (ortholog of *Drosophila 14-3-3ζ*) RNAi by 23% and 43% for *E. coli* and *S. aureus*, respectively (Fig. 1C, right panel). These results indicate that *Abi*, *cpa*, and *14-3-3ζ/Ywhaz* have evolutionarily conserved roles in the phagocytosis of bacteria. Of these genes, *14-3-3ζ/Ywhaz*, a member of a conserved, ubiquitous signaling adaptor protein family, has not been studied previously in the context of microbial phagocytosis and was thus chosen to be characterized further.

To gain more insight about the role of 14-3-3ζ in phagocytosis, we first investigated whether it is required for cell surface binding of bacteria, the first step in the phagocytic process. We performed a flow cytometry-based phagocytosis assay at +4°C. At this temperature, cells bind but do not internalize bacteria. RNAi, against the phagocytosis receptor Eater, was used as a positive control and *SCAR* dsRNA, which affects internalization but not bacterial binding [26], as a negative control. Under these conditions, a dsRNA treatment targeting *14-3-3ζ* did not affect the amount of S2 cell-associated, FITC-labeled *E. coli* or *S. aureus* (data not shown), indicating that it affects the internalization rather than the binding of bacterial particles.

14-3-3ζ RNAi flies are compromised in their ability to phagocytose *E. coli* ex vivo and in vivo

To investigate the in vivo relevance of 14-3-3ζ for phagocytosis in *Drosophila*, we used a larval hemocyte phagocytosis assay [5, 26]. *14-3-3ζ* RNAi flies were crossed over the *Hml*$^{\Delta}$*-GAL4* driver line to target the expression of the RNAi construct to the hemocytes. RNAi flies crossed over w^{1118} were used as controls. Primary hemocytes were isolated from wandering third instar larvae, and their ability to internalize FITC-labeled *E. coli* was investigated using fluorescence microscopy. Primary hemocytes from induced *14-3-3ζ* RNAi flies internalized approximately one-tenth of the FITC-labeled, heat-killed *E. coli* compared with hemocytes from uninduced *14-3-3ζ* RNAi flies ($P=5.4\times10^{-5}$; **Fig. 2A** and **B**). *Eater* RNAi flies were used as positive controls and showed severely impaired phagocytosis, whereas hemocytes from the *Hml*$^{\Delta}$*-GAL4* driver line flies phagocytosed *E. coli* normally (Fig. 2A and B). These results indicate that hemocytes from *14-3-3ζ* RNAi flies are compromised in their ability to phagocytose heat-killed *E. coli* ex vivo, thus validating our in vitro findings.

To further confirm the role of 14-3-3ζ in *Drosophila* phagocytosis, we studied the ability of *Drosophila* plasmatocytes to internalize heat-killed *E. coli* using an in vivo phagocytosis assay [5, 31]. pHrodo-conjugated, heat-killed *E. coli* (25 nl) were injected into *14-3-3ζ* RNAi flies and into uninduced control flies and incubated for 30 min, after which, the flies were mounted onto agarose plates for analysis. Under these conditions, sessile hemocytes around the dorsal vessel, visualized by *Hml*$^{\Delta}$*-GAL4*-driven GFP expression in **Fig. 3A**, phagocytose injected microbes [5, 31] (Fig. 3A–D). As shown earlier [31], phagocytosis of heat-killed *E. coli* can be eliminated with preinjection of latex beads (Fig. 3E). Similarly, engulfment of bacteria is prevented at +4°C (Fig. 3F). *Eater* null flies, which are deficient in recognition of *E. coli* [5], were used as a positive control (Fig. 3G). As shown in Fig. 3H–K, knocking down *14-3-3ζ* by RNAi in vivo using a hemocyte driver (*Hml*$^{\Delta}$*-GAL4*) decreased the phagocytic index by more than 50%. This indicates that *14-3-3ζ* expression is needed in plasmatocytes for normal phagocytosis of heat-killed *E. coli* in *Drosophila* in vivo.

14-3-3ζ/Ywhab is essential for bacterial phagocytosis in zebrafish

To study whether the role of *14-3-3ζ/Ywhab* in phagocytosis is conserved from insects to vertebrates in vivo, we used live imaging to assess the ability of zebrafish leukocytes to recognize, bind, and phagocytose bacteria in WT and *14-3-3ζ/Ywhab* knockdown larvae. First, MO oligonucleotides were injected at the one to four cell stage, followed by an injection of FITC-labeled *E. coli* into the blood circulation at 2 dpf. As shown in **Fig. 4A** and **B**, leukocytes of zebrafish larvae are able to bind fluorescently labeled *E. coli* (Fig. 4A) and *S. aureus* (Fig. 4B). In contrast, we were unable to locate any leukocyte-associated, FITC-labeled *E. coli* or *S. aureus* in *14-3-3ζ/Ywhab* MO-injected

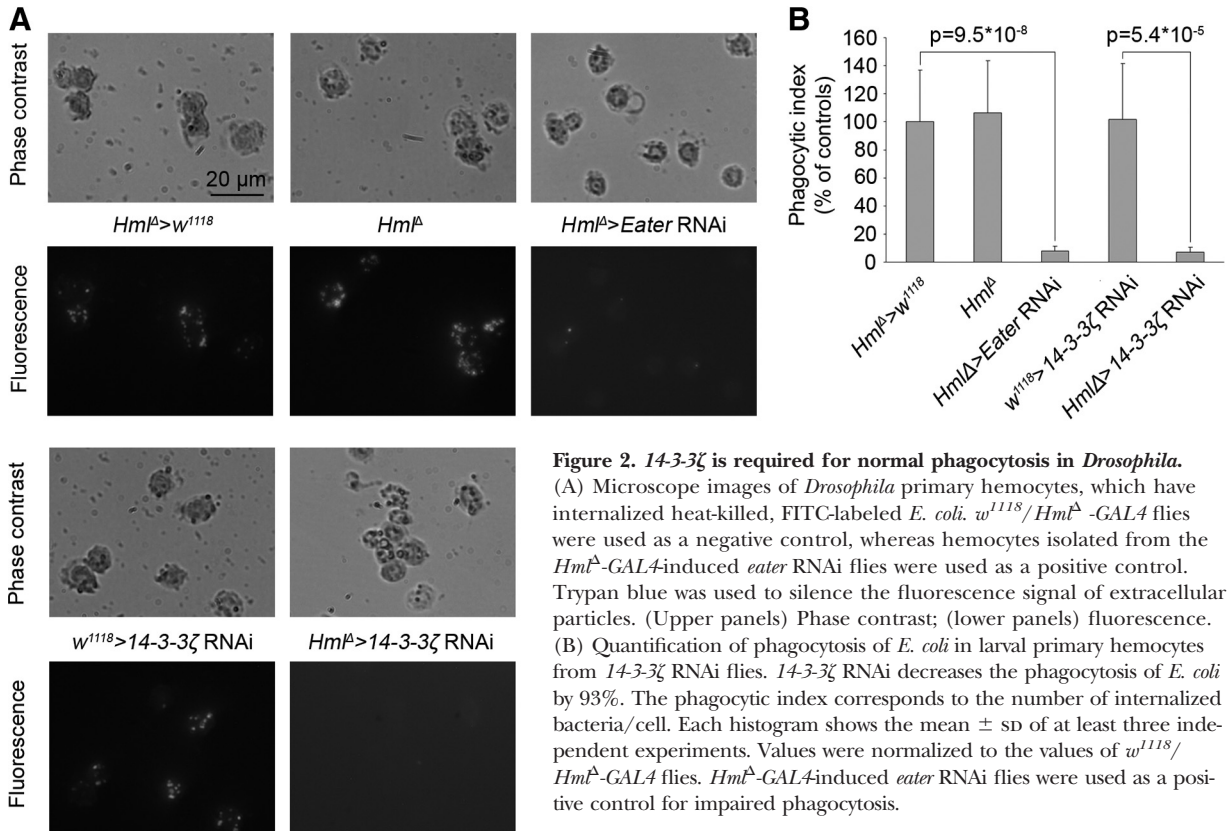

Figure 2. *14-3-3ζ* is required for normal phagocytosis in *Drosophila*. (A) Microscope images of *Drosophila* primary hemocytes, which have internalized heat-killed, FITC-labeled *E. coli*. w^{1118}/Hml^{Δ} *-GAL4* flies were used as a negative control, whereas hemocytes isolated from the Hml^{Δ}*-GAL4*-induced *eater* RNAi flies were used as a positive control. Trypan blue was used to silence the fluorescence signal of extracellular particles. (Upper panels) Phase contrast; (lower panels) fluorescence. (B) Quantification of phagocytosis of *E. coli* in larval primary hemocytes from *14-3-3ζ* RNAi flies. *14-3-3ζ* RNAi decreases the phagocytosis of *E. coli* by 93%. The phagocytic index corresponds to the number of internalized bacteria/cell. Each histogram shows the mean ± SD of at least three independent experiments. Values were normalized to the values of w^{1118}/Hml^{Δ}*-GAL4* flies. Hml^{Δ}*-GAL4*-induced *eater* RNAi flies were used as a positive control for impaired phagocytosis.

larvae (data not shown). This notion prompted us to develop an accurate method for measuring the rate of phagocytosis by zebrafish leukocytes in vivo.

To quantify the phagocytic ability of leukocytes, we designed a novel, quantitative in vivo phagocytosis assay for the zebrafish model system. Three hours after bacterial injections, the zebrafish larvae were dissociated, and the phagocytosed bacteria were quantified from the cells by FACS analysis. Specific hematopoietic cell populations cannot be distinguished in a total cell population of dissociated zebrafish larvae based on cell size and granularity. Therefore, we first gated a population based on size to include 1–2% of the total cell population. To confirm that the gated cell population contained the phagocytic cells, we targeted two MOs against pu.1, a transcription factor essential for the development of myeloid cells [27]. MO targeting of pu.1 led to the abolishment of FITC-positive cells with internalized fluorescent bacteria (Fig. 4C), indicating that the measured FITC signal corresponds specificically to the myeloid cell population. To further validate this in vivo phagocytosis assay, we targeted three genes known to be required for bacterial phagocytosis: *WASp1*, *WASp2*, and *Abi2* (Fig. 4D and E). WASp family proteins are key transducers of signals to the actin cytoskeleton and essential for phagocytic mechanisms [32]. A combination of two MOs targeting zebrafish WASp1 and WASp2 [23] caused a 69% reduction in bacterial phagocytosis (P=0.00027; Fig. 4D). Abi2 is an interactor of Abelson kinase, a signaling molecule regulating cytoskeletal organization, cell shape, and motility. Abi2 is a component of the SCAR/WAVE, a conserved regulatory complex required for the formation of dynamic protrusions [33–35]. There are two copies of the *Abi2* gene (Abi2a: Ensembl transcript ID ENSDART00000081015, and Abi2b: Ensembl transcript ID ENSDART00000081014) in the zebrafish genome. MOs were designed against both genes, and based on the in vivo phagocytosis assay, it turned out that one of the two copies (*Abi2a*, hereafter called *Abi2*) was a functional ortholog of the *Drosophila Abi* (Fig. 4E; data not shown). After Abi2 MO treatment, bacterial phagocytosis was reduced by 43% (P=0.0017; data not shown). These results confirmed that our novel in vivo zebrafish phagocytosis assay is a valid method for studying the roles of specific genes in bacterial phagocytosis.

Next, we used this new assay to analyze the importance of *14-3-3ζ/Ywhab* for the phagocytosis of FITC-labeled *E. coli* in zebrafish embryos. Injection of a *14-3-3ζ/Ywhab* translation-blocking MO (MO1) caused an 83 ± 12% reduction in phagocytosis (Fig. 4F and H; $P=8.2\times10^{-3}$). To confirm the specificity of the result, we designed a second, splice-blocking MO (MO2) against *14-3-3ζ/Ywhab*, which led to a similar, 87 ± 15% decrease in phagocytic ability (Fig. 4G and H; $P=8.0\times10^{-3}$). Sequencing of the altered *14-3-3ζ/Ywhab* transcript from MO2-injected larvae revealed an insertion of the intron 3-4, leading to 17 scrambled aa after exon 3 and a premature stop codon (Supplemental Fig. 1). Regardless of the observed phagocytosis phenotype with MO2 MO, RT-PCR analysis showed a remaining, strong band representing the WT transcript, in addition to the altered transcript (Fig. 4I). The WT transcript could not be eliminated by increasing the MO dose, as this caused high mortality of morphant embryos. These results indicate that *14-3-3ζ/Ywhab* is essential for bacte-

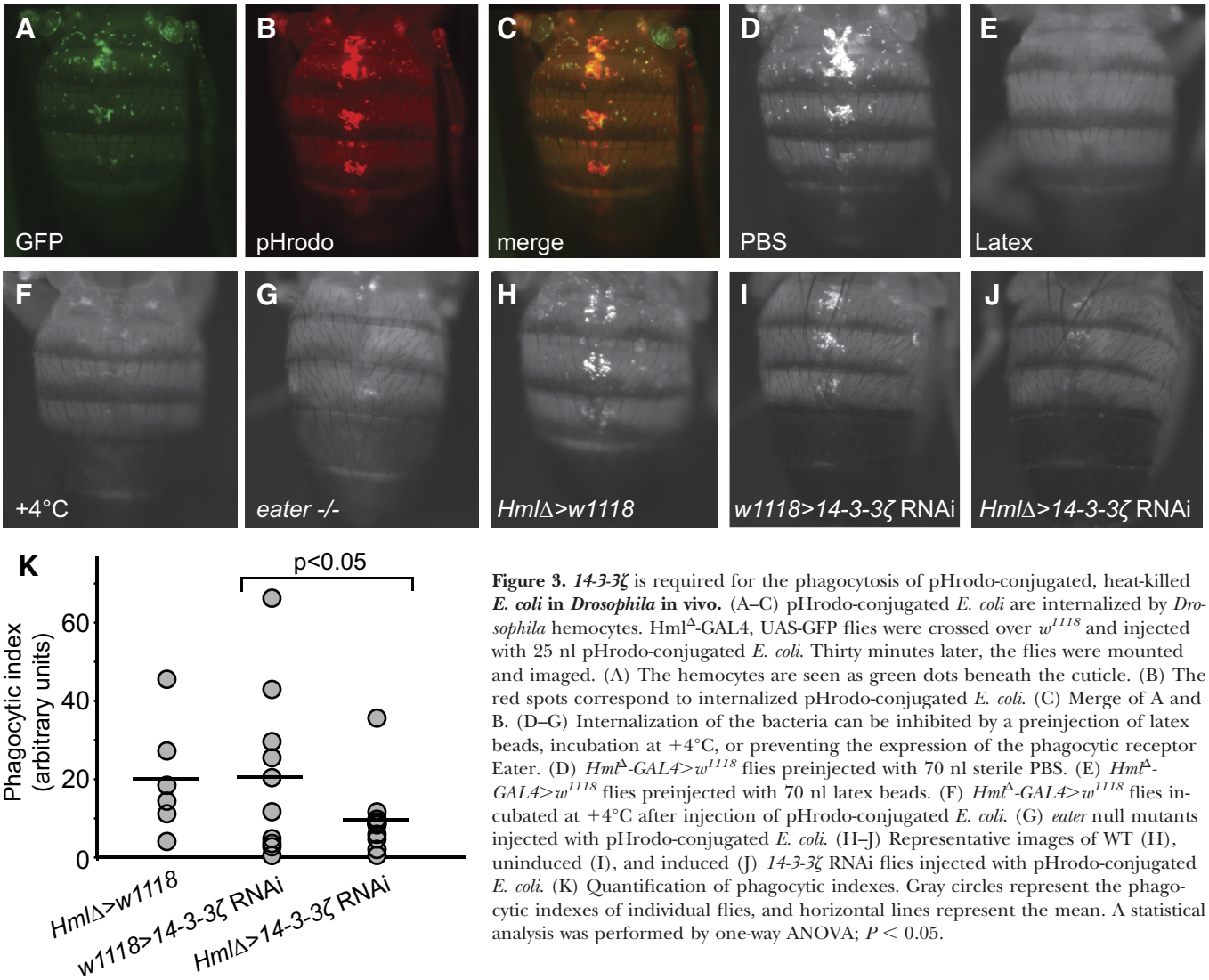

Figure 3. ***14-3-3ζ*** is required for the phagocytosis of pHrodo-conjugated, heat-killed ***E. coli*** **in** ***Drosophila*** **in vivo.** (A–C) pHrodo-conjugated *E. coli* are internalized by *Drosophila* hemocytes. Hml$^{\Delta}$-GAL4, UAS-GFP flies were crossed over w^{1118} and injected with 25 nl pHrodo-conjugated *E. coli*. Thirty minutes later, the flies were mounted and imaged. (A) The hemocytes are seen as green dots beneath the cuticle. (B) The red spots correspond to internalized pHrodo-conjugated *E. coli*. (C) Merge of A and B. (D–G) Internalization of the bacteria can be inhibited by a preinjection of latex beads, incubation at +4°C, or preventing the expression of the phagocytic receptor Eater. (D) *Hml$^{\Delta}$-GAL4>w^{1118}* flies preinjected with 70 nl sterile PBS. (E) *Hml$^{\Delta}$-GAL4>w^{1118}* flies preinjected with 70 nl latex beads. (F) *Hml$^{\Delta}$-GAL4>w^{1118}* flies incubated at +4°C after injection of pHrodo-conjugated *E. coli*. (G) *eater* null mutants injected with pHrodo-conjugated *E. coli*. (H–J) Representative images of WT (H), uninduced (I), and induced (J) *14-3-3ζ* RNAi flies injected with pHrodo-conjugated *E. coli*. (K) Quantification of phagocytic indexes. Gray circles represent the phagocytic indexes of individual flies, and horizontal lines represent the mean. A statistical analysis was performed by one-way ANOVA; $P < 0.05$.

rial phagocytosis in zebrafish and that its phagocytic role is conserved from insects to vertebrates in vivo.

14-3-3ζ/Ywhab is essential for surviving a *S. aureus* infection in *Drosophila* and zebrafish

Phagocytosis plays an important role in resistance against septic *S. aureus* infection in *Drosophila* [6, 7]. To this end, we studied the role of 14-3-3ζ in *S. aureus* infection. The expression of *14-3-3ζ* was suppressed by in vivo RNAi, by crossing two different *14-3-3ζ* RNAi stocks with the hemocyte-specific *Hml$^{\Delta}$-GAL4* driver line. The same RNAi flies, crossed over w^{1118} (uninduced RNAi), served as negative controls. The flies were pricked with a tungsten needle previously dipped into the bacterial suspension, and the survival of the infected flies was monitored for 144 h (**Fig. 5A**). During this time, suppression of *14-3-3ζ* with the 17870 R-2 RNAi construct gave a drastic decrease in survival compared with the control flies ($P=4.7\times10^{-6}$). The survival with the *14-3-3ζ* R-1 RNAi construct was also impaired significantly ($P=0.0007$). To exclude the possibility that the *14-3-3ζ* RNAi flies had died because of a general immune deficiency or reduced viability, we caused a septic infection with *E. cloacae*, which is effectively resisted without phagocytosing hemocytes [7]. Importantly, *14-3-3ζ* RNAi flies were capable of resisting the infection as well as WT flies (Fig. 5B), indicating normal viability and humoral immune response. As expected, more than 50% of the Relish^{E20} mutant flies, which have an impaired humoral immune response as a result of a lack of the Imd pathway transcription factor Relish, died within 40 h after infection. Importantly, antimicrobial peptide expression via the Toll and the Imd pathways was normal in *14-3-3ζ* RNAi flies upon microbial infection (Supplemental Fig. 2). This rules out the possibility that the susceptibility of *14-3-3ζ* RNAi flies to a *S. aureus* infection would be a result of an altered humoral immune response. In conclusion, these results indicate that the cytoskeletal regulatory protein *14-3-3ζ* is required for normal resistance against septic *S. aureus* infection in *Drosophila* in vivo.

Finally, we determined whether *14-3-3ζ/Ywhab* is required for zebrafish to mount an effective immune response against *S. au-*

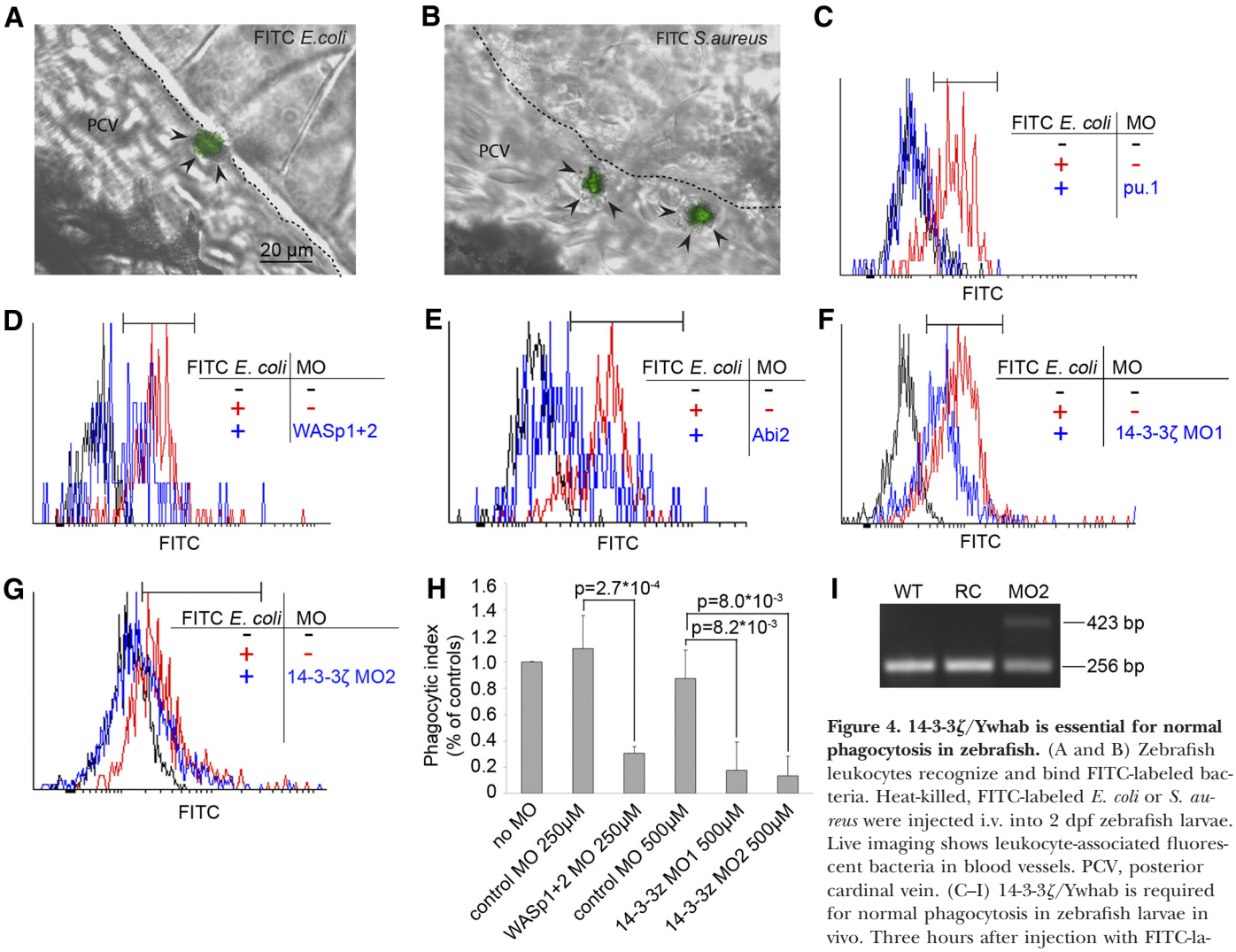

Figure 4. 14-3-3ζ/Ywhab is essential for normal phagocytosis in zebrafish. (A and B) Zebrafish leukocytes recognize and bind FITC-labeled bacteria. Heat-killed, FITC-labeled *E. coli* or *S. aureus* were injected i.v. into 2 dpf zebrafish larvae. Live imaging shows leukocyte-associated fluorescent bacteria in blood vessels. PCV, posterior cardinal vein. (C–I) 14-3-3ζ/Ywhab is required for normal phagocytosis in zebrafish larvae in vivo. Three hours after injection with FITC-labeled *E. coli*, the MO-treated and control larvae were dissociated into single cells, and the internalized fluorescent bacteria were quantified using flow cytometry. (C) Pu.1 MO led to the complete abolishment of FITC-positive events representing bacterial phagocytosis (D and E). Silencing of *Abi2* and *WASp1 + 2*, characterized genes required for the phagocytic mechanism, resulted in the reduction of internalized bacteria (F–H). Injections with two independent MOs targeting 14-3-3ζ/Ywhab decrease phagocytosis. After MO injections targeting *14-3-3ζ/Ywhab*, the uptake of fluorescent *E. coli* was reduced by 37%. The phagocytic index was calculated by multiplying the percentage of FITC-positive cells by the MFI of these cells. Each histogram represents the mean ± SD of three or more independent experiments. (I) The splice-blocking MO (MO2) against *14-3-3ζ/Ywhab* leads to an alteration in the size of the transcript (Lane 3, upper band), caused by insertion of an intron. RC, random control.

reus. The *S. aureus* infection model was chosen, as it has been shown that a myeloid cell response, including phagocytosis, is essential for the immune defense and survival during systemic *S. aureus* infection in zebrafish larvae [36]. *14-3-3ζ/Ywhab* was targeted in all tissues of zebrafish larvae by MO injections. One thousand six hundred cfu *S. aureus* were microinjected into the blood circulation of 2 dpf zebrafish to study the survival rates of the MO-treated larvae. MO targeting of *14-3-3ζ/Ywhab* reduced the survival of larvae compared with controls ($P<0.0001$; Fig. 5C). Similarly, survival rates of larvae injected with WASp1 + 2 MOs were significantly lower, and all of the larvae died within 50 h postinfection ($P<0.0001$; Fig. 5C). 14-3-3ζ/Ywhab and especially WASp1 + 2 MOs also caused a higher mortality in buffer (0.2 M KCl)-injected larvae, but the effect was more subtle compared with *S. aureus*-challenged morphants (Fig. 5D). Our results suggest that MO targeting of 14-3-3ζ/Ywhab affects myeloid cells (directly or indirectly via other tissues) and impairs their ability to phagocytose bacteria. Furthermore, these results suggest that disrupting the ability of myeloid cells to phagocytose leads to poor survival of zebrafish larvae during septic *S. aureus* infection. As a mediator of the phagocytosis of bacteria, 14-3-3ζ/Ywhab is essential for the normal immune defense and survival in a vertebrate zebrafish model (Fig. 5C).

DISCUSSION

Drosophila S2 cells are well suited for RNAi screening because of the rapid receptor-mediated internalization of dsRNA directly from the cell culture medium [21]. In recent years, several RNAi

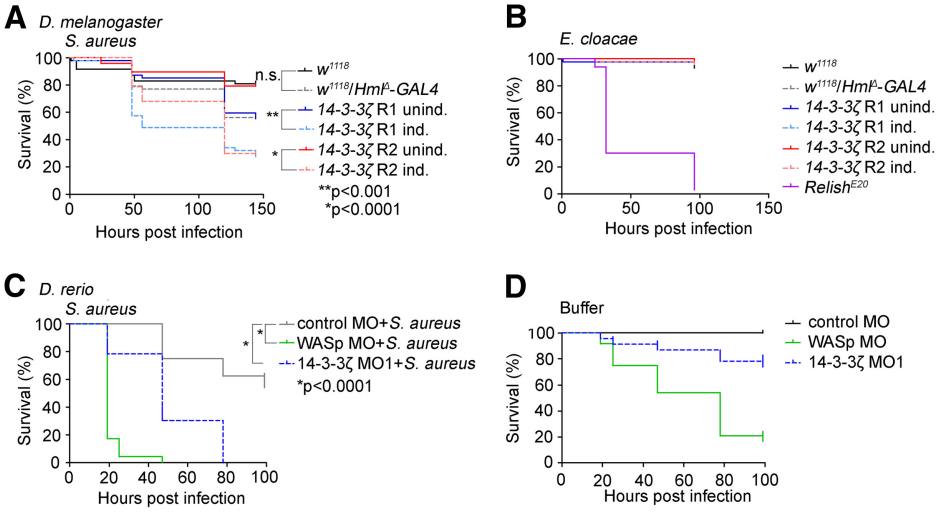

Figure 5. Silencing of 14-3-3ζ increases susceptibility to *S. aureus* infection in *Drosophila* and zebrafish in vivo. (A) Induced (ind.) *14-3-3ζ* RNAi flies R-1 and R-2 (*Hml*$^{\Delta}$*-GAL4>14-3-3ζ* RNAi) have significantly lower survival rates compared with uninduced (unind.; w^{1118}*>14-3-3ζ* RNAi) RNAi flies and control flies w^{1118} and w^{1118}/*Hml*$^{\Delta}$ *-GAL4* in septic *S. aureus* infection. Flies (n=43–48) were pricked with a needle contaminated with *S. aureus*, and survival was monitored for 144 h. The graph shows one representative experiment. (B) *14-3-3ζ* RNAi flies resisted *E. cloacae* septic injury in a manner comparable with that of control flies, indicating a normal humoral immune response. *Relish*^{E20} flies were used as a positive control. Survival after *E. cloacae* infection was monitored for 96 h; n = 45–48. (C) *14-3-3ζ/Ywhab* expression is required for normal resistance to septic *S. aureus* infection in zebrafish larvae. After MO treatment, 2 dpf zebrafish larvae were injected i.v. with 1600 cfu *S. aureus*. The survival of the larvae was followed for 96 h. After 14-3-3ζ/Ywhab1 MO treatment, the survival of zebrafish larvae was reduced (P<0.0001). *D. rerio*, *Danio rerio*. (D) i.v. injection of 0.2 M KCl did not cause mortality in the control MO-injected larvae. Survival rates of 14-3-3ζ/Ywhab and especially WASp1 + 2 MO-injected larvae were decreased by the trauma caused by the buffer injection. In both cases, however, the effect on survival was more severe upon infection with *S. aureus*. The graphs show one representative experiment; n = 21–24 in each group.

screens aiming to identify gene products involved in phagocytosis have been published [1, 10, 11, 16, 17, 37]. So far, there are limited functional data about most of the identified genes, and in most cases, the in vivo relevance of these in vitro findings remains to be analyzed. In the current study, we used RNAi screening to identify new mediators of bacterial phagocytosis. By screening 3048 dsRNA treatments, we found six gene products, which play an elementary role in the internalization of *E. coli* and *S. aureus*, representatives of Gram-negative and Gram-positive bacteria, respectively. These were Abi (CG9749), cpa (CG10540), signaling adaptor 14-3-3ζ (CG17870), tlk (CG2829), and the unnamed gene products CG2765 and CG15609.

The *Drosophila* gene *CG2765* encodes a protein of unknown function. However, its human homologue, MIDI interacting G12-like protein, has been shown to function as a stabilizer of microtubular structures [38]. *CG15609* encodes a predicted actin-binding protein. The closest mouse homologue of CG15609 is Ehbp1, which contains a putative actin-binding calponin homology domain. Together with its interacting partner EHD2, Ehbp1 has been implicated in endocytosis [39]. In addition, EHD2 contains a domain that possibly links it to the actin cytoskeleton, namely, a putative Arp2/3 complex-binding motif. siRNA silencing of these genes inhibits transferrin endocytosis in cultured 3T3-L1 fibroblasts, whereas overexpression of *EHD2* and *EHBP1* leads to extensive actin reorganization in COS-1 cells. Thus, these proteins have been proposed to provide a functional linkage between the plasma membrane and actin filaments, thus connecting endocytosis to the cytoskeleton [39].

Importantly, we were able to demonstrate that Abi, cpa, and 14-3-3ζ have evolutionarily conserved roles in phagocytosis. Of these three proteins, Abi and cpa have been identified previously as mediators of the phagocytosis of the yeast *Candida albicans* [17], and the bacteria *Mycobacterium fortuitum* and *Listeria monocytogenes* in *Drosophila* cells [11, 16], whereas 14-3-3ζ has not been studied earlier in the context of bacterial phagocytosis. Here, we show that 14-3-3ζ has an important role in phagocytosis in *Drosophila* in vivo and that it is required for normal resistance against *S. aureus* in *Drosophila*.

For *Drosophila*, knowledge of the role of phagocytosis in the resistance against microbial infections has been limited, but recent studies have elucidated the role of phagocytosis in *Drosophila* host defense. In 2005, the transmembrane protein Eater was identified as a mediator of bacterial phagocytosis [5]. Moreover, *Eater* null flies had defects in their immune response and increased susceptibility to bacterial infection, although they displayed a normal, NF-κB-mediated antibacterial peptide response. More recently, it was shown that elimination of *Drosophila* hemocytes by apoptosis resulted in an increased susceptibility to *Enterococcus faecalis* and *S. aureus* infections [6, 7], whereas resistance to *E. cloacae* was comparable with that of WT flies [7]. Similarly to the Eater mutant flies, apoptotic targeting of *Drosophila* hemocytes in either of the above-mentioned studies did not affect the production of antimicrobial peptides via the Imd or Toll pathways, indicating a normally functioning, humoral immune response and providing further evidence for the importance of phagocytosis in *Drosophila* host defense. In line with these findings, we show that depletion of 14-3-3ζ expression in the hemocytes of RNAi knockdown flies results in impaired phagocytosis and a reduced ability to fight against septic *S. aureus* infection but does not compromise AMP production. This indicates that the phagocytic activity of plasmatocytes (i.e., not only the recognition of pathogenic bacteria) is required for a normal innate host defense in *Drosophila*.

Zebrafish is a vertebrate model organism with a fully developed immune system, including adaptive and innate immunity. Importantly, the MO gene-silencing technique, which is effective up to 5 dpf, can be used in zebrafish larval infection models. At this developmental stage, the adaptive immunity is still absent, and phagocytes play a central role in the host immune response. In

this study, we developed a flow cytometry-based method for accurately quantifying zebrafish phagocytosis in vivo and showed that *14-3-3ζ/Ywhab* expression is required for the normal phagocytosis of heat-killed, fluorescent *E. coli* in zebrafish embryos in the myeloid blood cell compartment. Consistent with the earlier reports about the importance of phagocytosis for the immune defense against bacterial infections in the zebrafish model [27, 36, 40], *14-3-3ζ/Ywhab* was shown to be essential for survival during a *S. aureus* infection. Our findings indicate that the zebrafish is a valid model for studying the relevance of specific genes for bacterial phagocytosis and the immune defense in vivo and that the role of *14-3-3ζ/Ywhab* is functionally conserved from insects to vertebrates.

14-3-3ζ belongs to the 14-3-3 proteins. Two 14-3-3 proteins (ε and ζ) have been identified in *Drosophila* and at least seven isoforms in mammals. The mouse and zebrafish orthologs of 14-3-3ζ are 14-3-3ζ/Ywhaz and 14-3-3ζ/Ywhab. 14-3-3 family members are cytosolic proteins capable of forming homo- and heterodimers [41]. 14-3-3 proteins are known to participate in several cellular and physiological processes, including neuronal functions [42], the regulation of cell cycle [43], apoptosis [44], and exocytosis [45, 46]. 14-3-3 proteins also function with MAPKAPKs in controlling cytoskeletal architecture, inflammation, and malignant growth [47]. Typically, their molecular function is to regulate signal transduction by interacting with their enzyme ligands in a phosphoserine-dependent manner [41, 48]. In these interactions, the 14-3-3 dimer functions as an adaptor protein, which determines the spatial organization of the signaling complex, modulates enzyme activity, or determines the subcellular localization of cargo proteins. In the cytoskeletal context, 14-3-3 proteins have been suggested to interact with several regulators of the actin cytoskeleton and/or cellular movements. Especially, 14-3-3 proteins have been characterized in the regulation of the actin depolymerizing factor/cofilin (Supplemental Fig. 3). Cofilin is essential for the dynamic, cellular movements, which facilitate changes in the actin cytoskeleton. In its active, dephosphorylated form, cofilin disassembles F-actin from the pointed ends of the actin filament network to recycle actin monomers to the leading edge for further rounds of polymerization (Supplemental Fig. 3). Cofilin activity is controlled by phosphorylation. The ubiquitous LIM kinases have been shown to be primarily responsible for cofilin phosphorylation and deactivation, whereas the SSH family of proteins has been suggested to dephosphorylate and subsequently activate cofilin [49] (Supplemental Fig. 3). According to the current literature, 14-3-3 is capable of interacting with the cofilin kinase LIM and the cofilin phosphatase SSH, as well as with cofilin itself [50, 51] (Supplemental Fig. 3). Interestingly, none of the previous genome-scale RNAi screens for phagocytosis identified 14-3-3ζ or its regulatory target, cofilin. However, according to Stuart et al. [52], 14-3-3ζ is one of the proteins enriched in phagosome preparations isolated from *Drosophila* S2 cells, suggesting a role in phagocytosis. In our study set-up, depletion of the expression of 14-3-3ζ in S2 cells using RNAi resulted in a 47 ± 20% and a 44 ± 15% reduction in phagocytosis of *E. coli* and *S. aureus*, respectively, whereas the same values for the RNAi of the *Drosophila* cofilin homologue twinstar were 54 ± 2% and 60 ± 3%, respectively (data not shown). In a study by Delorme et al. [53], the enhanced global, long-term activation of cofilin resulted in increased actin-filament elongation and reduced protrusion efficiency. This elegant study defines cofilin as a central switch for controlling the lamellipodium, lamella, and adhesions in response to upstream signals. According to Delorme et al. [53], the cofilin-orchestrated signaling network must be tightly controlled to transform the F-actin assembly into dynamic cell protrusions. Indeed, in our S2 cell model, RNAi targeting of cofilin or its negative regulator 14-3-3ζ led to strongly impaired phagocytosis of bacteria, thereby demonstrating the need of dynamic changes in the formation of protrusions required for efficient phagocytosis. As overexpression of *14-3-3ζ* has been shown to lead to the accumulation of inactive, phosphorylated cofilin, as a result of its protection from phosphorylation [54], we hypothesize that *14-3-3ζ* RNAi releases cofilin for dephosphorylation and subsequent activation, leading to excessive actin polymerization and the prevention of cellular protrusions in response to bacterial stimulus (Supplemental Fig. 3). This defines cofilin regulator C as an essential positive regulator of actin rearrangements, leading to cell protrusions and bacterial phagocytosis under immune challenge.

In conclusion, our results indicate that 14-3-3ζ is an important mediator of phagocytosis in *Drosophila* in S2 cells and primary plasmatocytes ex vivo and in vivo. In addition, the role of 14-3-3ζ in phagocytosis is evolutionarily conserved, as depletion of its expression in mouse macrophage-like RAW 264.7 cells, as well as in zebrafish larvae in vivo, resulted in strongly impaired phagocytic function. 14-3-3ζ also plays a significant role in the *Drosophila* and zebrafish host defense against *S. aureus* infection. Altogether, these results demonstrate the power of *Drosophila* S2 cell-based RNAi screening to identify evolutionarily conserved and thus, central gene products that are important for a normal, immune response in invertebrates as well as in vertebrates.

AUTHORSHIP

J.U. performed the primary screen and most of the in vitro experiments. L-M.V., A.K., and C.K. performed the in vivo fly experiments. J.U., M.V-M., and M.H. performed the RAW 264.7 cell experiments. J.U., M.V., and M.P. performed the zebrafish experiments. J.U. and S.E. carried out the confocal microscopy. All authors cowrote the paper.

ACKNOWLEDGMENTS

This work was supported by grants from the Sigrid Juselius Foundation (M.H. and M.R.), the Academy of Finland (M.R., D.H., and M.P.), the Foundation for Pediatric Research (M.R.), Tampere Tuberculosis Foundation (M.R.), the Emil Aaltonen Foundation (M.R.), the Swedish Research Council and the Swedish Cancer Society (D.H.), and Competitive Research Funding of the Pirkanmaa Hospital District (M.R. and M.P.). The zebrafish work was done at University of Tampere Zebrafish Core Facility, funded by Biocenter Finland. The fly work was done at University of Tampere Drosophila Core Facility, funded by Biocenter Finland. We thank Heidi Enwald, Tapio Kotipelto, Mirkka Ovaska, and Dr. Samuli Rounioja for technical assistance and members of the M.H. and M.R. labs for stimulating discussions. In addition, we thank NIG-Fly and VDRC for RNAi fly lines.

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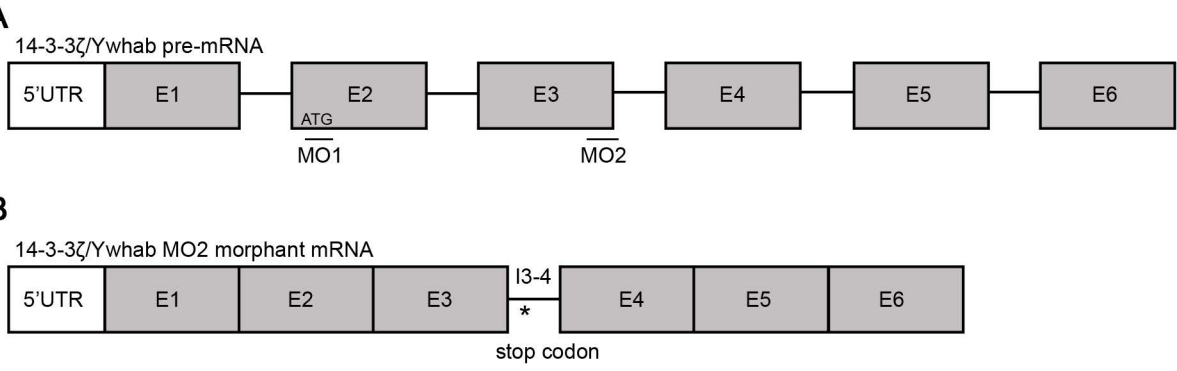
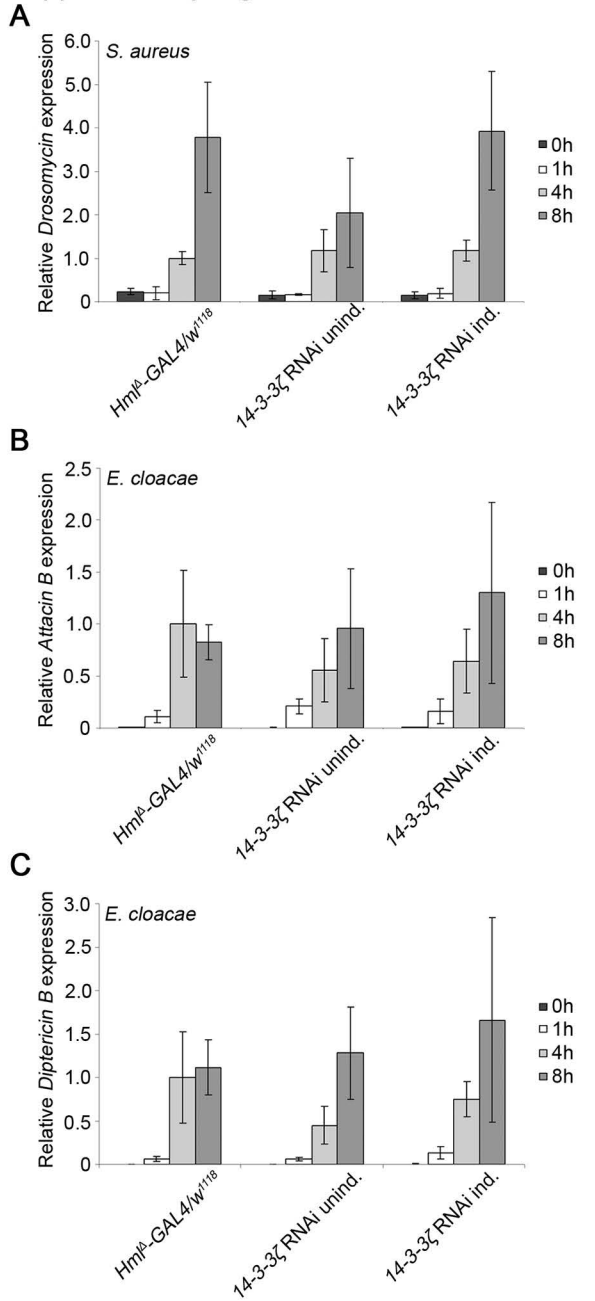
KEY WORDS:
Drosophila · zebrafish · plasmatocyte · macrophage · immunity

Supplementary Figure 1



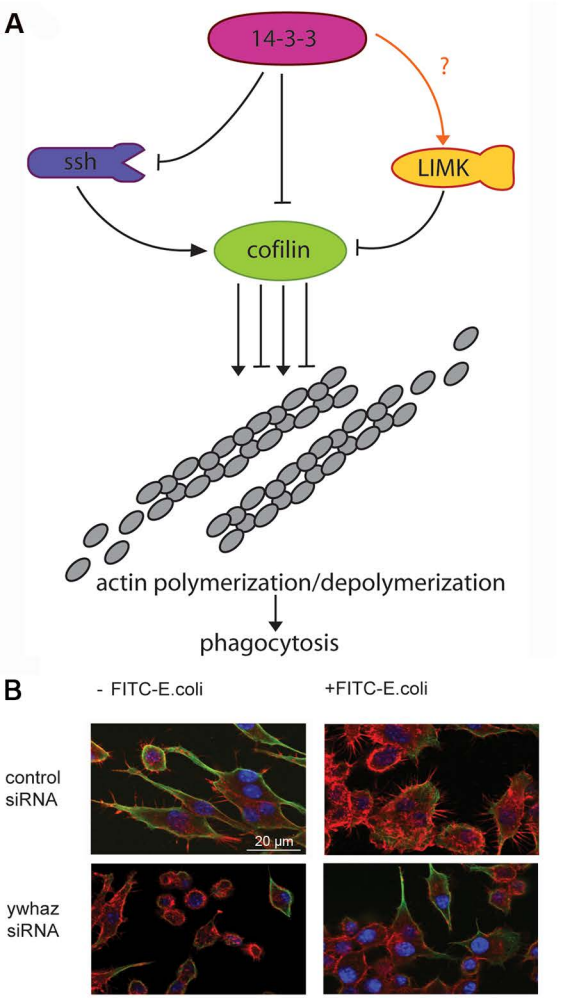
Supplementary Figure 1. Morpholino targeting of 14-3-3ζ in zebrafish embryos.
(A) MO1 translation blocking morpholino targets the 5' UTR region at the translation initiation site. MO2 is designed to bind the splice donor site at the exon4 - intron3-4 boundary.
(B) Binding of MO2 into the 14-3-3 pre-mRNA interferes with the splicing and leads to the inclusion of the intron3-4 in the transcript. Translation of the altered transcript leads to 17 scrambled amino acids after the exon3, and truncation of the protein due to a premature stop codon (asterisk).

Supplementary Figure 2



Supplementary Figure 2. 14-3-3ζ RNAi flies have normal AMP expression. (A-C) *w*^{1118}*/Hml*$^{\Delta}$*-GAL4, w*^{1118}*>14-3-3ζ* RNAi (uninduced) and *Hml*$^{\Delta}$*-GAL4>14-3-3ζ* RNAi (induced) flies were infected with *S. aureus* (A) (to induce Toll pathway signaling) or *E. cloacae* (B,C) (to induce Imd pathway signaling) and the expression levels of *Drosomycin* (A), *Attacin B* (B) and *Diptericin B* (C) were analyzed by qRT-PCR from total RNAs extracted at indicated time points after infection.

Supplementary Figure 3



Supplementary Figure 3. 14-3-3ζ is an essential regulator of actin rearrangements

(A) A schematic presentation of 14-3-3ζ as a regulator of cofilin. Cofilin is essential for facilitating dynamic changes in the actin cytoskeleton. The activity of cofilin is controlled by phosphorylation. Dephosphorylated (active) cofilin disassembles F-actin from the pointed ends of the actin filament network to recycle actin monomers to the leading edge for further rounds of polymerization. LIM kinases have been shown to phosphorylate (deactivate) cofilin and the Slingshot (SSH) to dephosphorylate cofilin. According to the current literature, 14-3-3ζ interacts with LIM and SSH, as well as with cofilin itself.

(B) Depletion of *14-3-3ζ/Ywhaz* releases cofilin for dephosphorylation leading to excessive actin polymerization and the prevention of cellular protrusions in response to bacterial stimulus. RAW 264.7 cells were transfected with control and *14-3-3ζ/ywhaz* siRNAs and were allowed to phagocytoze heat-killed FITC-labelled *E.coli.* The cells were then stained with a DAPI nuclear stain (blue), anti-β-tubulin (green) and phalloidin (red). The cytoskeletal staining shows that the inhibition of *14-3-3ζ/ywhaz* with siRNAs results in excessive actin polymerization (lower left) and lack of cellular protrusions after encountering with bacteria (lower right), as compared to control siRNA treated cells (upper left and right).

Table I Targeted RNAi treatments decreasing cell viability

GENE INFORMATION			PHAGOCYTOSIS (% of control±SD)		VIABILITY		
					β-gal	z-score	
GENE	NAME	FUNCTION	*E. coli*	n		$S2R^+$	Kc_{167}
CG2807	-	mRNA splicing	12±17	6	**0.52**	**6.1**	**4.3**
CG16941	-	mRNA splicing	5±3	7	1.33	**3.8**	1.6
CG2925	*noisette*	mRNA splicing	31±9	4	**0.43**	**3.5**	2.1
CG8144	*pasilla*	mRNA splicing	20±15	5	0.96	**3.4**	1.6
CG1913	*α-Tubulin at 84B*	Cytoskeletal, GTPase	23±21	6	1.18	**3.4**	0.6
CG2512	*α-Tubulin at 84D*	Cytoskeletal, GTPase	18±8	3	0.84	**3.2**	0.4
CG3992	*serpent*	Transcription factor	13±7	5	**0.32**	**3.1**	**4.8**
CG6292	*Cyclin T*	Cdk regulator	50±22	4	**0.40**	**3.1**	**3.7**
CG7439	*Argonaute 2*	RNA interference	26±10	4	**0.07**	2.9	1.5
CG4147	*Hsc70-3*	Protein folding	44±22	11	**0.53**	2.9	2.9
CG11198	-	Fatty acid biosynthesis	36±20	4	1.03	2.8	**3.3**
CG1528	*γCop*	Vesicle transport	59±17	4	**0.56**	2.4	-0.3
CG8522	*HLH106*	Transcription factor, Fatty acid biosynthesis	14±13	8	**0.54**	2.3	**4.3**
CG6699	*β'Cop*	Vesicle transport	13±8	7	**0.49**	2.0	0.4
CG11624	*Ubiquitin-63E*	Ribosomal	23±18	15	**0.12**	**5.9**	**7.7**
CG3523	-	Fatty acid biosynthesis	13±21	13	**0.45**	-0.4	0.9
CG6815	*belphegor*	Nucleoside-triphosphatase	54±34	4	**0.32**	-0.3	-0.4
CG1639	*lethal (1) 10Bb*	Transcriptional regulation	54±46	5	**0.67**	0.7	0.4
CG5887	*desat1*	Fatty acid biosynthesis	9±9	6	**0.45**	1.1	**4.6**
CG7269	*Helicase at 25E*	mRNA splicing	44±6	4	**0.39**	1.0	1.9
CG17255	-	Unknown	42±20	3	**0.21**	0.4	-1.1
CG4389	-	Fatty acid beta-oxidation	50±29	3	**0.65**	0.2	0.5
CG2331	*TER94*	Endopeptidase, ATPase	45±22	6	**0.72**	0.3	1.5
CG11763	*midlife-crisis*	Transporter	57±32	7	**0.73**	0.1	-0.1
CG5271	*RpS27A*	Ribosomal	24±3	3	**0.15**	1.9	1.0
CG8877	*prp8*	mRNA splicing	27±31	6	**0.68**	1.5	0.4
CG5119	*pAbp*	polyA-binding protein, Translational regulation	47±9	4	-	**3.0**	**3.6**

β-gal value ≤0.75 and z-score ≥3.0 (z-scores from [29])
were considered to indicate decreased cell viability (bold values).

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Functional Characterization of the Infection-Inducible Peptide Edin in *Drosophila melanogaster*

Leena-Maija Vanha-aho[1☯], Anni Kleino[1☯], Meri Kaustio[1], Johanna Ulvila[2], Bettina Wilke[1], Dan Hultmark[1,3], Susanna Valanne[1], Mika Rämet[1,4]*

1 BioMediTech and Institute of Biomedical Technology, University of Tampere, Tampere, Finland, **2** Department of Pediatrics, and Biocenter Oulu, University of Oulu, Oulu, Finland, **3** Department of Molecular Biology, Umeå University, Umeå, Sweden, **4** Department of Pediatrics, Tampere University Hospital, Tampere, Tampere, Finland

Abstract

Drosophila is a well-established model organism for studying innate immunity because of its high resistance against microbial infections and lack of adaptive immunity. In addition, the immune signaling cascades found in Drosophila are evolutionarily conserved. Upon infection, activation of the immune signaling pathways, Toll and Imd, leads to the expression of multiple immune response genes, such as the antimicrobial peptides (AMPs). Previously, we identified an uncharacterized gene edin among the genes, which were strongly induced upon stimulation with Escherichia coli in Drosophila S2 cells. Edin has been associated with resistance against Listeria monocytogenes, but its role in Drosophila immunity remains elusive. In this study, we examined the role of Edin in the immune response of Drosophila both in vitro and in vivo. We report that edin expression is dependent on the Imd-pathway NF-κB transcription factor Relish and that it is expressed upon infection both in vitro and in vivo. Edin encodes a pro-protein, which is further processed in S2 cells. In our experiments, Edin did not bind microbes, nor did it possess antimicrobial activity to tested microbial strains in vitro or in vivo. Furthermore, edin RNAi did not significantly affect the expression of AMPs in vitro or in vivo. However, edin RNAi flies showed modestly impaired resistance to E. faecalis infection. We conclude that Edin has no potent antimicrobial properties but it appears to be important for E. faecalis infection via an uncharacterized mechanism. Further studies are still required to elucidate the exact role of Edin in the Drosophila immune response.

Citation: Vanha-aho L-M, Kleino A, Kaustio M, Ulvila J, Wilke B, et al. (2012) Functional Characterization of the Infection-Inducible Peptide Edin in *Drosophila melanogaster*. PLoS ONE 7(5): e37153. doi:10.1371/journal.pone.0037153

Editor: François Leulier, French National Centre for Scientific Research – Université Aix-Marseille, France

Received December 20, 2011; **Accepted** April 15, 2012; **Published** May 14, 2012

Funding: This research was supported by grants from the Academy of Finland, the Sigrid Juselius Foundation (to MR), the Tampere Tuberculosis Foundation (to MR and SV), Competitive Research Funding of the Pirkanmaa Hospital District (to MR and SV), the Tampere Graduate Program in Biomedicine and Biotechnology (LMV) and the Orion-Farmos Research Foundation (LMV). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: Mika.Ramet@uta.fi

☯ These authors contributed equally to this work.

Introduction

Innate immunity is the first line of defense in all multicellular organisms. During the last few decades, the fruit fly *Drosophila melanogaster* has proven to be well suited for studying innate immune responses. In contrast to vertebrates, *Drosophila* only has an innate immune system, which is highly sophisticated and in part conserved among higher organisms [1]. In *Drosophila*, effective innate immune responses are based on the ability of several pattern-recognition receptors to recognize and bind common microbial surface structures. One main outcome of this initial microbial recognition is the activation of NF-κB immune signaling pathways, which leads to the production of several potent antimicrobial peptides (AMPs).

In *Drosophila*, the production of AMPs is mainly regulated by two NF-κB signaling pathways: the Imd (immune deficiency) pathway [2] reviewed in [3] and the Toll pathway [4] reviewed in [5]. Both of these pathways are highly conserved from fly to man. The Imd pathway is activated by diaminopimelic acid-type peptidoglycan (DAP) [6], present in most or all Gram-negative bacteria, but also in some Gram-positive bacteria like *Listeria monocytogenes*. The Toll pathway is activated mainly by the lysine-type peptidoglycan present in many other Gram-positive bacteria [7], reviewed in [5]. Both of these signaling pathways can also be induced by different fungi [8,9]. Activation of the Imd and Toll signaling pathways upon microbial infection ultimately causes the nuclear translocation of the NF-κB transcription factors, Relish or Dif/Dorsal respectively, leading to the expression of dozens of NF-κB responsive genes [10,11,12,13,14]. The molecular function of many of these genes still remains unknown.

Earlier, we identified a gene, *CG32185*, to be highly induced in S2 cells in response to heat-killed *Escherichia coli* [14]. Later, Gordon et al. called the gene *edin* and found it to be associated with *Listeria monocytogenes* resistance [15]. In addition, it has been shown that Edin is secreted into the hemolymph in *Drosophila* third instar larvae upon infection [16]. Because the molecular function of Edin and the signaling pathways involved are still mainly unknown, in our current study we set out to examine the role of Edin in the *Drosophila* immune response both *in vitro* and *in vivo*.

Results

Edin expression is Relish-dependent *in vitro* and *in vivo* upon Gram-negative bacterial infection

When *Drosophila* encounters microbes, several signaling pathways are activated leading to transcriptional modifications. This response varies depending on the microbe and the site of infection. During a systemic infection, the expression of dozens of genes is induced [11,12] leading to very effective defense responses. Upon infection, most of the highly induced genes are known to be *AMP* genes, *DIM*s (Drosophila immune-induced molecules) or genes related to signal regulation. Nevertheless, the molecular function of several of the induced genes is yet to be characterized. Previously, we studied which genes are induced in response to heat-killed *Escherichia coli* in *Drosophila* macrophage-like S2 cells [14]. Table I represents the oligonucleotide microarray data of the most strongly induced genes (data collected from [14]). The eight most strongly induced genes encode five known AMPs, one peptidoglycan recognition protein (*PGRP-LB*), a negative regulator of the Imd pathway (*pirk*) [17] and *edin* (CG32185). According to the microarray results, the expression of *edin* is strongly induced within hours after the bacterial challenge and the induction pattern of *edin* resembles that of known antimicrobial peptides (Table I).

In S2 cells, the response to *E. coli* is known to be predominantly mediated via the Imd pathway [13]. To verify whether the induction of *edin* is dependent on the Imd pathway, we silenced the Imd pathway by knocking down the transcription factor Relish by RNAi. The induction of *edin* was completely abolished in *Relish* dsRNA treated S2 cells at the 4 h time point (Table I) indicating that *edin* expression is regulated via the Imd pathway in S2 cells after induction with heat-killed *E. coli*.

The *edin* gene encodes a short peptide of 115 amino acids including an N-terminal signal sequence (amino acids 1–22) (Figure 1A). The predicted signal peptidase cleavage site is supported by proteomic data from Verleyen et al. [16], who identified the predicted amino terminal of the mature protein in peptide fragments from hemolymph. Likely orthologs of the *edin* gene can be found in other brachyrecan flies, including all sequenced *Drosophila* species, but not in other insects (Figure 1A). For *Musca domestica*, three isoforms are represented in the EST databases (not shown). A tendency for pseudogenisation of the *edin* genes can be noted, as stop codons are present in the *D. yakuba* and *D. mojavensis* homologs. For the latter, an apparently functional allele is represented by an EST sequence (Figure 1A). A stop codon interrupts the open reading frame in the EST from *Lucilia sericata*, but this could be a sequencing error.

Iterated PSI-BLAST searches indicate that Edin is related to the Attacin/Diptericin superfamily of glycine-rich antibacterial peptides. The best hits were to *Drosophila virilis* Diptericin B (E = 8e-20) and *Hyalophora cecropia* Attacin E (E = 2e-18). Figure 1A shows an alignment to Diptericin B and the C-terminal (G2) domain of Attacin A from *D. melanogaster*.

Since Edin has a predicted signal sequence, we next examined if Edin is actually secreted from cells. To test this, we cloned *edin* cDNA into the heavy metal-inducible expression vector pMT/V5, transfected S2 cells with the construct and analyzed the presence of the protein both in the cell culture medium and cell extracts by western blotting using an anti-V5 antibody. In the S2 cells, both shorter and longer forms of Edin were detected, corresponding to V5-tagged peptides with and without the signal sequence, respectively. In the cell culture medium, only the shorter, C-terminal form, without the signal sequence could be observed (Figure 1B). This result suggests that Edin has a functional signal sequence, which is cleaved before the peptide is secreted. These results are in line with the report of Verleyen and coworkers [16], who detected amino-terminal fragments of Edin with mass spectrometry in the hemolymph of *Drosophila* larvae infected with a mixture of Gram-negative and Gram-positive bacteria.

Since the expression of *edin* is Relish-dependent *in vitro*, we next investigated whether *edin* is also induced upon microbial challenge *in vivo*. We infected wild-type *Canton S* and *Relish* null mutant adult flies (Rel^{E20}) with the Gram-negative bacteria *Enterobacter cloacae*. Total RNAs were extracted and the transcript levels of *edin* were determined with RT-PCR and agarose gel electrophoresis. As shown in Figure 1C, *edin* is induced in *Canton S* but not in Rel^{E20} mutant flies. *Attacin A* was used as a positive control and showed a similar expression pattern to *edin* (Figure 1C). These results together with the previously published microarray data indicate that *edin* expression is strongly and rapidly induced upon a Gram-negative bacterial infection in a Relish-dependent manner both *in vitro* and *in vivo*. These results together propose that Edin has a function related to microbial resistance. Thus, we next subjected Edin to further functional characterization both *in vitro* and *in vivo*.

Edin has no significant effect on bacterial binding

The phagocytosis of invading microbes is an essential component of *Drosophila* immunity [18,19]. To this end we tested whether Edin has a role in bacterial binding or opsonization. Plasmatocyte-like S2 cells that are capable of binding and phagocytosing microbes [20] were treated with *edin* dsRNA and the ability of the cells to bind heat-killed, fluorescently labeled *E. coli* and *Staphylococcus aureus* was analyzed with flow cytometry. As a positive control, we used a dsRNA treatment targeting *eater*, which

Table 1. Induction of *Drosophila* antimicrobial peptide genes and *edin* in *E. coli* -challenged S2 cells (data collected from [14]).

Gene	#CG	0 h	0.5 h	1 h	4 h	24 h	*Relish* RNAi 4 h
Attacin B	CG18372	1±0.1	1.5	6.0	60.6±15.1	87.2±87.2	0.1±0.0
Diptericin B	CG10794	1±0.0	2.3	3.6	52.4±4.3	78.1±1.8	0.2±0.1
Attacin D	CG7629	1±0.0	1.1	2.6	47.5±6.3	92.5±2.1	0.1±0.1
Metchnikowin	CG8175	1±0.0	1.7	6.5	41.3±15.7	52.2±2.0	0.4±0.1
Edin	CG32185	1±0.1	0.9	3.5	29.8±6.4	48.5±1.1	0.0±0.0
Pirk	CG15678	1±0.2	2.0	15.5	15.1±0.6	5.4±0.0	0.4±0.1
PGRP-LB	CG14704	1±0.0	1.2	2.0	8.5±2.0	20.7±0.3	0.7±0.2
Cecropin B	CG1878	1±0.0	1.2	3.2	7.4±0.9	4.0±0.3	0.6±0.0

doi:10.1371/journal.pone.0037153.t001

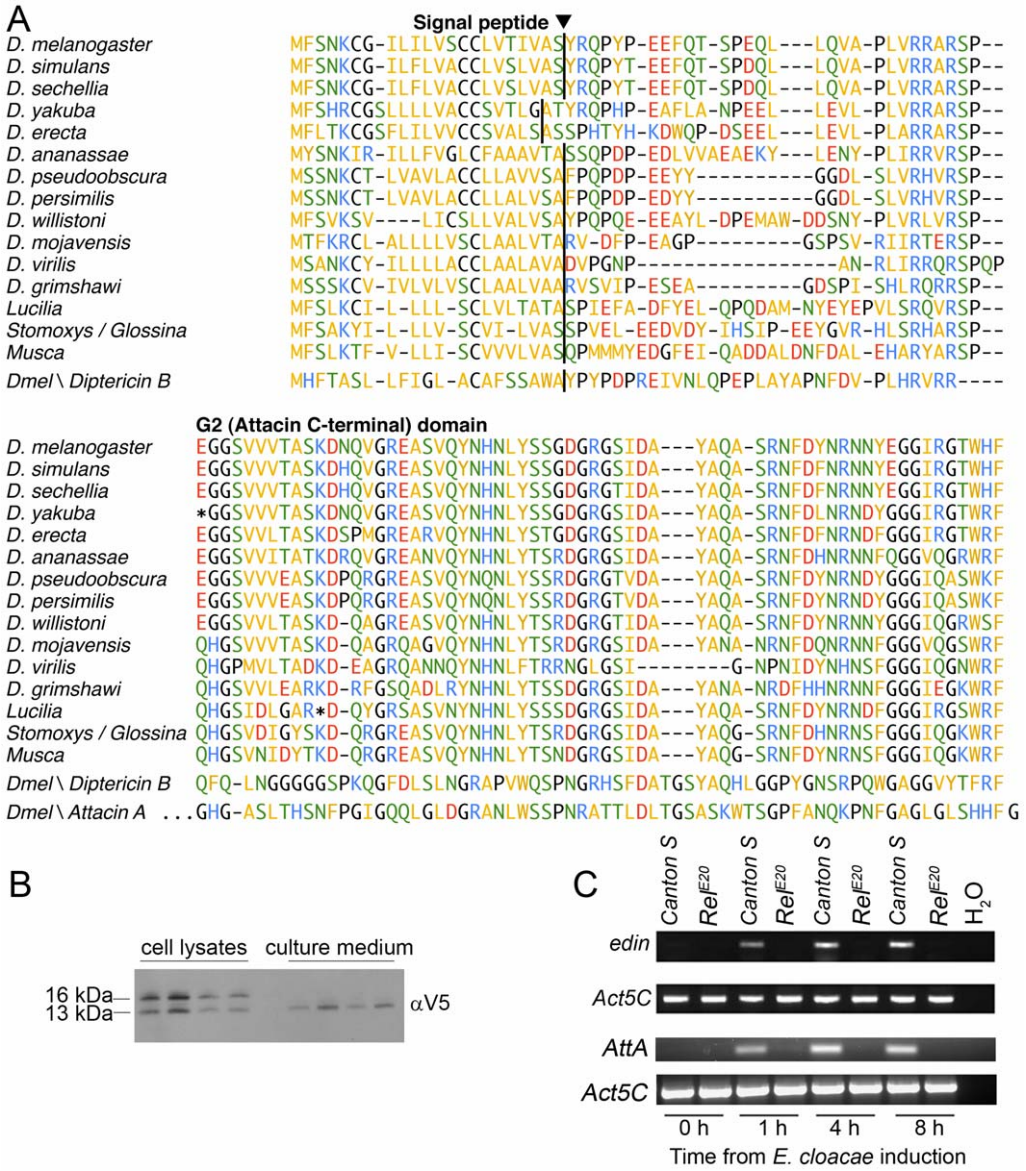

Figure 1. Edin is a Relish-dependently synthesized peptide, which is secreted from S2 cells. (A–B) Edin contains a signal sequence and is secreted from S2 cells. (A) Edin sequences are aligned from 12 *Drosophila* species and three other dipterans. Diptericin B and Attacin A from *D. melanogaster* are also included in the alignment. The predicted signal peptidase cleavage sites [31] are marked. The sequences from the 12 *Drosophila* species are all from Clark et al. 2007 [32], except the *D. mojavensis* sequence which is derived from an EST sequence (EB600147). Modified gene models without introns were used for *D. yakuba* and *D. willistoni*. The *Lucilia sericata* sequence is derived from a single EST (FG360503). Three *Stomoxys calcitrans* ESTs (DN952426, DN952940, EZ048833) and one *Glossina morsitans* EST (AF368915) appear to contain overlapping sequence from the same gene. The *Musca domestica* sequence is an isoform represented by one EST (ES608713). (B) The signal sequence of Edin is cleaved before the peptide is secreted to the cell culture medium. S2 cells were transfected with a pMT-*edin*-V5 construct and the cell culture medium and cell lysates were analyzed with western blotting. Both full-length and cleaved forms were observed in the lysates while only the cleaved form was present in the medium. The V5 tag is located at the C-terminus of Edin. The blot represents 4 independent samples from which both cell lysates and culture medium were analyzed. (C) *Edin* is induced upon *Enterobacter cloacae* infection in *Canton S* flies but not in Rel^{E20} flies. Canton S flies and Rel^{E20}-mutant flies were pricked with *E. cloacae* and total RNAs were extracted at the indicated time points. RT-PCR was performed and samples were electrophoresed on an agarose gel. *Actin5C* was used as a loading control and *Attacin A* as a positive control.
doi:10.1371/journal.pone.0037153.g001

codes for an important phagocytic receptor for bacteria both in S2 cells and in *Drosophila in vivo* [18,19,21]. *GFP* dsRNA was used as a negative control. *Edin* RNAi did not affect the ability of S2 cells to bind *E. coli* (Figure 2A). Likewise, *edin* dsRNA treatments did not compromise the ability of S2 cells to bind *S. aureus* (Figure 2B) but rather seemed to modestly enhance the binding activity of S2 cells.

To test the effect of *edin* overexpression on bacterial binding, S2 cells were first transiently transfected with a pMT[*edin*]V5 construct. An empty pMT/V5 plasmid was transfected as a control. 24 h after transfection, $CuSO_4$ was added to the cell culture medium to induce the expression of the construct. Two days later, the medium was collected and transferred to other S2 cells which were pre-treated with *edin* dsRNA to block endogenous *edin* expression. Thereafter, FITC-labeled, heat-killed *E. coli* or *S. aureus* were added and the amount of cell-associated bacteria was monitored using flow cytometry. In line with the results of *edin*

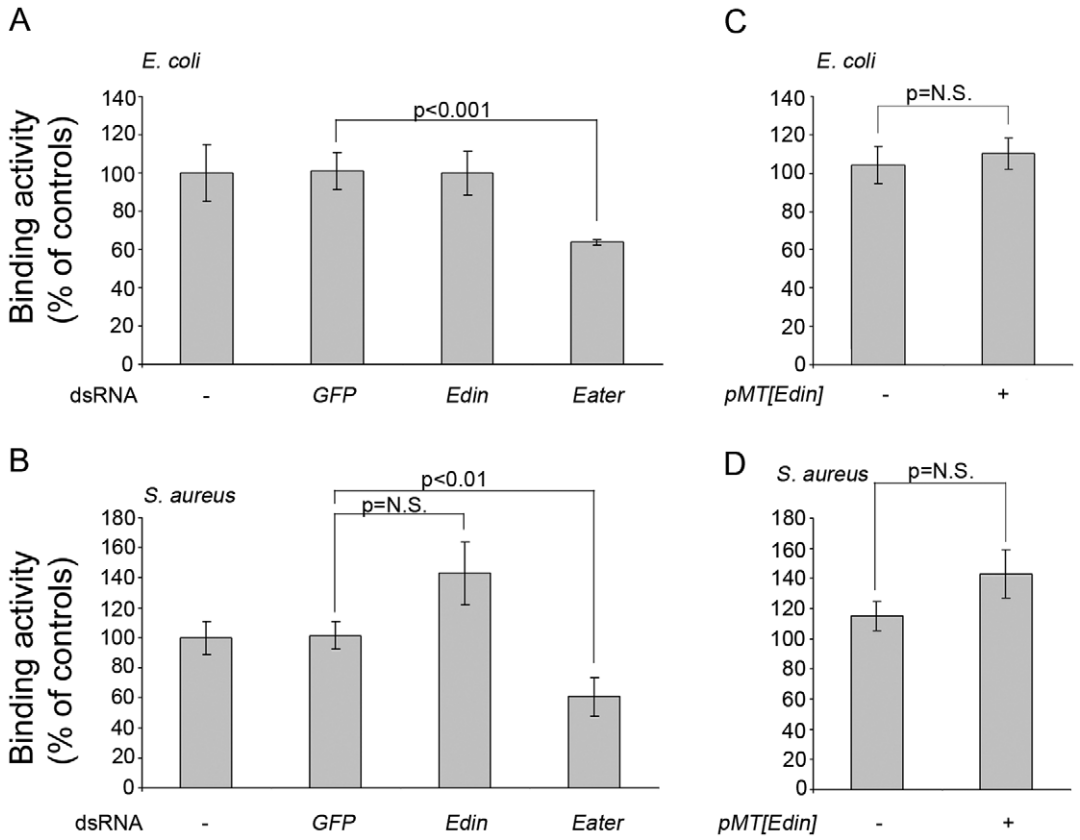

Figure 2. Edin does not affect the ability of S2 cells to bind microbes. (A–B) The effect of *edin* RNAi on the binding of *E. coli* and *S. aureus* in *Drosophila* S2 cells. *Drosophila* S2 cells were soaked for three days in dsRNAs and thereafter exposed to bacteria at +4°C. *GFP* dsRNA was used as a negative and *eater* dsRNA as a positive control. (C–D) The effect of *edin* overexpression on the binding of *E. coli* and *S. aureus.* S2 cells were transiently transfected with a pMT construct expressing *edin* and endogenous *edin* expression was knocked down with dsRNA treatments. The ability of S2 cells to bind heat-killed *E. coli* (A, C) or *S. aureus* (B, D) was measured using flow cytometry.
doi:10.1371/journal.pone.0037153.g002

RNAi experiments, *edin* overexpression had no effect on the binding of *E. coli* (Fig. 2C) or *S. aureus* (Fig. 2D). The presence of Edin in the cell-culture medium was confirmed by western blotting using an anti-V5 antibody (data not shown).

To investigate in a more direct way if Edin binds microbes, we incubated Edin-containing cell culture medium with live *E. coli*, *Serratia marcescens*, *Staphylococcus epidermidis*, *Enterococcus faecalis*, *Listeria monocytogenes*, *Micrococcus luteus*, *Saccharomyces cerevisiae* and *S. aureus*. Latex beads (carboxylated polystyrene), which are expected to bind all kinds of proteins to some extent, were used as a positive control. The microbial suspensions were incubated with 500 µl of Edin-containing medium at +4°C after which the microbes were pelleted and washed with PBS. Finally, the pellets were suspended and boiled in an SDS-PAGE sample buffer to detach bound Edin from the microbes before electrophoresis. Next, the proteins were transferred onto nitrocellulose membranes and Edin was detected using an anti-V5 antibody. As a reference, 20 µl of Edin-containing medium was loaded into the first lane. Therefore, if Edin attached efficiently to the indicated microbe, much more Edin should be detected in the samples (500 µl Edin-containing medium used) compared to the reference lane (20 µl Edin-containing medium). As shown in Figure 3 (the rightmost lanes), carboxylated latex beads, i.e. the positive control, bound Edin. In contrast, virtually no Edin was bound to the tested Gram-negative bacteria, *E. coli* and *S. marcescens*. Furthermore, only a faint signal was detected with the Gram-positive bacteria *S. epidermidis*, *E. faecalis*, *L. monocytogenes*, *M. luteus* and *S. aureus*, and with the baker's yeast *S. cerevisiae* as compared to the reference lane (ctrl in Figure 3). Based on these results, we conclude that Edin does not strongly bind any of the tested microbes.

The effect of Edin on immune signaling

Next, we investigated whether Edin is involved in modulating the activity of *Drosophila* innate immune signaling cascades. S2 cells were transfected with luciferase-reporter constructs together with *edin* dsRNA as well as with negative and positive control dsRNAs, and the luciferase activities of the cell lysates were analyzed. Transfection efficacy and cell viability were assessed with an *Actin 5C*-β-galactosidase reporter. *GFP* dsRNA was used as a negative control in all assays. First, we tested the effectiveness of *edin* RNAi *in vitro* by treating S2 cells with *GFP* or *edin* dsRNAs, and analyzing the relative expression levels of *edin*. As shown in Figure 4A, *edin* RNAi abolishes the endogenous *edin* expression.

In order to analyze the Imd pathway activity, an *Attacin A*-luciferase reporter and *Relish* dsRNA as a positive control were used and the pathway was activated by adding heat-killed *E. coli* to the cell culture medium. The samples were collected 0 h (no induction), 1 h, 4 h, 8 h and 24 h after *E. coli* induction. As expected, *Relish* RNAi strongly decreases the Imd-pathway activity at all time points (Figure 4B). On the contrary, *edin* RNAi had minor or no effect in this setting, although at the 24 h time point there was a trend for reduced *Attacin A* promoter driven luciferace

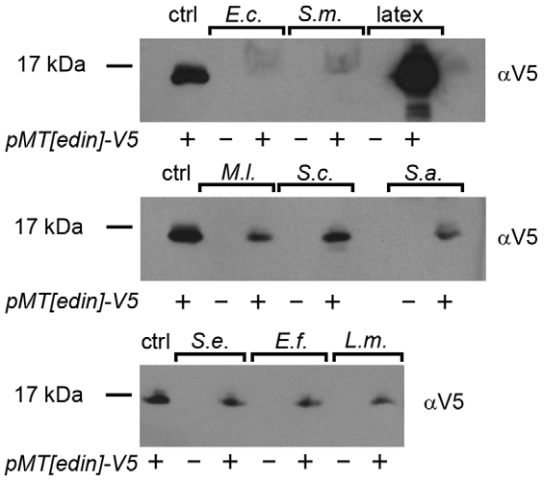

Figure 3. The effect of Edin on microbial binding. 500 µl of Edin-V5 containing medium were incubated with 1 ml of a bacterial suspension of live *E. coli* (E.c.), *Serratia marcescens* (S.m), *Staphylococcus epidermidis* (S.e.), *Enterococcus faecalis* (E.f.), *Listeria monocytogenes* (L.m.), *Micrococcus luteus* (M.l.), *Saccharomyces cerevisiae* (S.c.) or *S. aureus* (S.a) for 1 h with mild agitation at +4°C. Latex beads treated with BSA were used as a control. The samples were then centrifuged and the pellet was washed. Edin bound to microbes was detached by adding 20 µl of SDS-PAGE loading buffer, boiled for 10 minutes, electrophoresed on SDS-PAGE and detected using a V5 antibody. The first lane of each blot is a control sample containing 20 µl of Edin-V5 medium. The following lanes contain 30 µl of the medium incubated with the indicated microbe.
doi:10.1371/journal.pone.0037153.g003

activity (Figure 4B). Because *edin* RNAi appeared to have a minor effect on the Imd pathway activity when induced with heat-killed *E. coli* at the 24 h time point, we next investigated the effect of the *edin* dsRNA with other pathway elicitors. To this end, heat-killed *S. marcescens*, heat-killed *E. cloacae*, peptidoglycan and overexpression of the cytoplasmic tail of the PGRP-LC receptor were used. As shown in Figure 4C, *edin* RNAi had no effect on the *AttA*-luciferase activity in this experimental setting. These results indicate that Edin does not have an important role in the regulation of the Imd pathway activity in S2 cells.

To investigate the role of Edin in the Toll pathway signaling, we used a *Drosomycin*-luciferase reporter, and *MyD88* dsRNA as a positive control, and activated the pathway by transfecting the cells with a constitutively active form of the Toll receptor, $Toll^{10B}$ (Figure 4D) or with the cleaved, active Spätzle ligand (Figure 4E). For the JAK/STAT signaling pathway, we used *TurandotM*-luciferase reporter and *STAT* dsRNA as a positive control (Figure 4F). The pathway was activated by overexpressing $hopscotch^{Tum-l}$, the active form of *Drosophila* Jak. *Edin RNAi* did not significantly affect the signaling via the Toll pathway (Figure 4D–E), or the JAK/STAT pathway (Figure 4F). These results indicate that Edin has no central role in regulating immune signaling *in vitro*.

To test the role of Edin in Imd pathway regulation *in vivo*, we monitored the Imd pathway-mediated *AMP* gene expression levels with qRT-PCR in *edin* RNAi flies and in *edin* overexpression flies we created. The overexpression flies were created by microinjecting the *pUAST-edin* construct into Rel^{E20} mutant embryos. To analyze Imd pathway activity, *edin* RNAi (VDRC #14289) and *UAS-edin*,Rel^{E20} flies were crossed with the *C564-GAL4* driver that targets transgene expression to the fat body in addition to some other organs [22]. The Imd pathway was then activated in week-old offspring by septic injury with *E. cloacae*. Flies crossed with w^{1118} flies were used as controls. As shown in Figure 5A, *in vivo* RNAi of *edin* using the *C564-GAL4* driver strongly suppresses *edin* expression in whole flies, indicating that the UAS-RNAi construct is effective. *UAS-edin*,Rel^{E20} flies crossed with the *C564-GAL4* driver showed expression levels comparable to the *E. cloacae* infected control flies (Figure 5B).

In agreement with our *in vitro* results, *in vivo* RNAi of *edin* did not show any clear effect in the expression levels of the tested *AMP* genes (two left-most panels, Figure 5C–H). There is a trend towards a minor decrease at the 4 h time points of the tested *AMP*s, excluding *Drosocin* (Figure 5H), but the decrease was statistically significant only with *Cecropin A1* (Figure 5D) and *Attacin B* (Figure 5E). We next tested whether overexpression of *edin* affects the production of AMPs via the Imd pathway. We compared *AMP* expression after septic injury with *E. cloacae* between *UAS-edin* flies crossed with *C564-GAL4* and *UAS-edin* flies crossed with w^{1118} flies. We observed moderate increase only in *Drosocin* expression at the 8 h time point (68% increase for $p<0.05$) (Figure 5H). Noteworthy, *edin* expression did not activate *AMP* gene expression without a microbial challenge (see the 0 h time point in the rightmost panel in Figure 5C–H). This is in line with the results in S2 cells and rules out the possibility that Edin would function as a cytokine mediating immune response from the site of induction to other tissues (for example from hemocytes to the fat body). Based on these results, we conclude that Edin has no important role in the regulation of the Imd pathway activity either *in vitro* or *in vivo*.

Edin has no potent antimicrobial properties in vitro or in vivo

The kinetics of *edin* expression closely resembles those of known AMP genes, which led us to examine whether Edin has antimicrobial properties *in vitro* or *in vivo*. To study this, we first analyzed whether Edin was able to limit bacterial growth *in vitro*. We overexpressed *edin* in S2 cells, collected the cell culture medium and incubated the medium either with *E. coli* or *S. aureus*. Medium from S2 cells transfected with an empty vector was used as a control. As shown in Figure 6A and 6B, *E. coli* and *S. aureus* grew equally well in control medium and in medium containing Edin.

To further investigate the antimicrobial properties of Edin *in vitro*, we designed synthetic peptides containing the amino acids 22–45 (Edin C-terminal form) or 50–115 (Edin N-terminal form. The peptides were tested for their ability to reduce bacterial growth *in vitro*. Cecropin A and Lysozyme were used as positive controls for Gram-negative and Gram-positive bacteria, respectively. The peptides were incubated with *E. coli* (Figure 6C–D), *E. cloacae* (Figure 6E–F), *L. monocytogenes* (Figure 6G–H) or *E. faecalis* (Fig. 6I–J) and colony forming units were determined. As shown in Figure 6C–J, Cecropin A and Lysozyme at their highest concentrations almost abolished the growth of the tested microbes whereas neither the synthetic C-terminal or N-terminal form of Edin was able to affect the growth of the bacteria. Moreover, no synergistic effects were observed when Edin was incubated together with either Cecropin A or Lysozyme (three rightmost columns in Figure 6 panels C–J).

To test the antimicrobial properties of Edin in a more physiological context, the effect of *edin* overexpression on the survival of flies after bacterial infections was analyzed. First, to test whether overexpressing *edin* affects survival or lifespan, the *UAS-edin*,Rel^{E20} overexpression line was crossed with the *Act5C-GAL4/*CyO driver line and the lifespan of the offspring was monitored. As shown in Figure 7A, overexpression of *edin* did not affect the

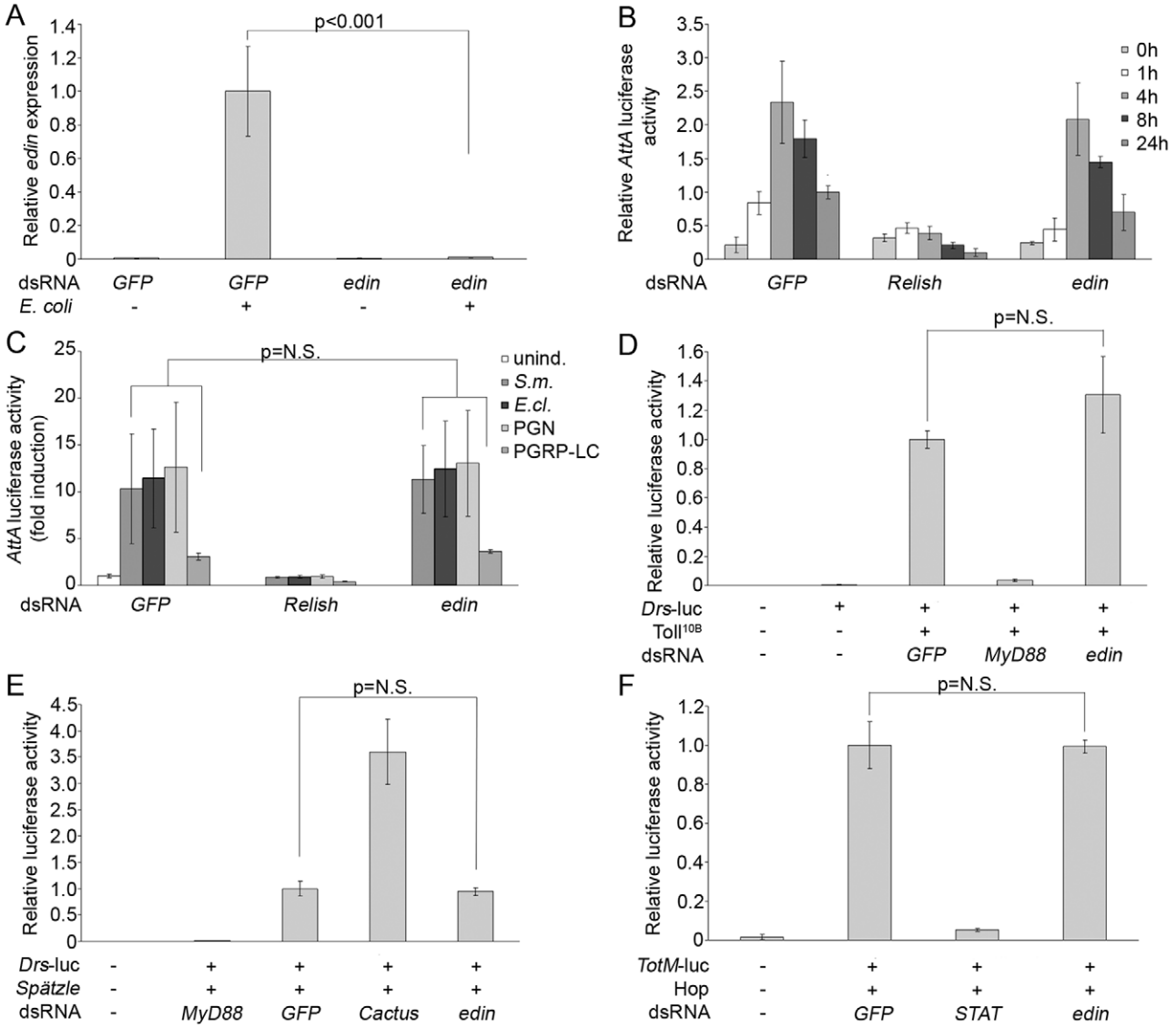

Figure 4. Effect of *edin* RNAi on *Drosophila* immune signaling in vitro. A) *Edin* RNAi is effective in S2 cells. S2 cells were treated with *GFP* and *edin* dsRNA and the cells were induced by adding heat-killed *E. coli*. Relative expression levels of *edin* were analyzed from total RNAs with qRT-PCR. n = 4 for each sample. (B) *Edin* expression is not required for the Imd pathway signaling *in vitro*. S2 cells were transfected with an *Attacin A*-luciferase reporter together with *GFP* (negative control), *Relish* (positive control) and *edin* dsRNAs. The Imd pathway was activated by adding heat-killed *E. coli* to the cell culture medium and samples were collected at indicated time points. *Edin* RNAi causes a 30% decrease in the Imd pathway activity at the 24 h time point. The data for the 0 h and 24 h time points are pooled from 5 indepent experiments (n = 17 per sample). For 1 h, 4 h and 8 h time points n = 4 per sample. (C) *Edin* RNAi does not decrease the Imd pathway activity when the pathway is induced with *S. marcescens*, *E. cloacae*, peptidoglycan or PGRP-LC. S2 cells were transfected with an *AttA*-luciferase reporter and *edin* dsRNA and the Imd pathway was activated with *S. marcescens* (*S.m.*), *E. cloacae* (*E.cl.*), peptidoglycan (PGN) or a *pMT[PGRP-LC]* construct. $CuSO_4$ was used to induce the expression of *PGRP-LC*. *GFP* and *Relish* dsRNAs were used as negative and positive controls, respectively. Unind. = no induction. The data for *S.m.*, *E.cl.* and PGN are pooled from 3 independent experiments (n = 12 per sample). For PGRP-LC, n = 3 per sample. (D) *Edin* RNAi does not affect the Toll pathway activity. S2 cells were transfected with a *Drosomycin*-luciferase reporter together with *GFP*, *edin* and *MyD88* (positive control) dsRNAs. A constitutively active form of the Toll receptor, $Toll^{10B}$, was used to activate the pathway. The data are pooled from 3 independent experiments, n = 10 for each sample. (E) *Edin* has no effect on the Spätzle-induced Toll-pathway activity. S2 cells were transfected with a *Drosomycin*-luciferase reporter together with *GFP*, *edin*, *MyD88* (control) and *Cactus* (control) dsRNAs. The Toll pathway was activated with the cleaved, active Spätzle ligand (Spz^{C106}). n = 4 for each sample. (F) *Edin* RNAi has no effect on the JAK/STAT pathway. S2 cells were transfected with a *Turandot M*-reporter and *GFP*, *STAT* (positive control) and *edin* dsRNAs. The JAK/STAT pathway was activated by overexpressing Hop^{Tum-l}. n = 4 for each sample.
doi:10.1371/journal.pone.0037153.g004

lifespan of the flies and was comparable to that of the control flies. Furthermore, *edin* expression did not compromise the development of flies since equal amounts of *UAS-edin,*Rel^{E20}*/ActGAL4* and *UAS-edin,*Rel^{E20}/CyO flies were obtained from the crosses (Figure 7B). Similar results were obtained when *edin* overexpression flies where crossed with either the *C564-GAL4* driver line or the ubiquitous *daughterless-GAL4* driver line (data not shown).

An earlier study has shown that the expression of a single AMP can restore antimicrobial activity in *Drosophila* [23]. To test whether the expression of *edin* is sufficient to enhance resistance against septic infection in adult flies, we expressed *edin* in a

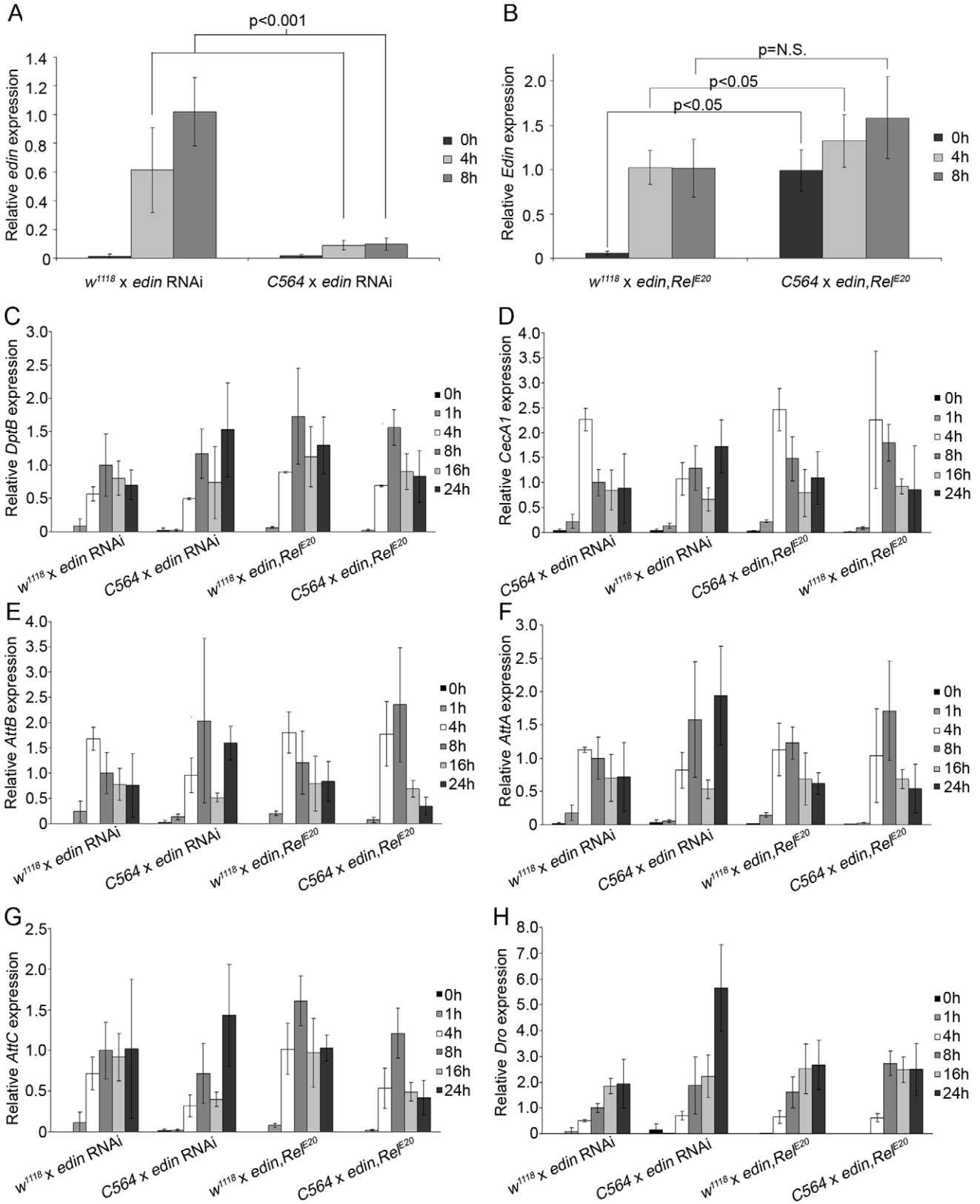

Figure 5. The effect of Edin on AMP production in vivo. *Edin* RNAi and overexpression flies (*edin,Rel*^{E20}) were crossed with *C564-GAL4* flies or w^{1118} flies as a control, their offspring was infected with *E. cloacae*, total RNAs were extracted at indicated time points and qRT-PCR for the indicated genes was performed. (A) Expression of *edin* is knocked down in *edin* RNAi flies crossed to *C564-GAL4* driver flies. (B) *Edin* overexpression flies express *edin* at a physiological level. *Edin* overexpression flies crossed with *C564-GAL4* have slightly higher levels of *edin* compared to flies crossed with w^{1118}. For (A–B) the data are pooled from 2 independent experiments, and n = 8 for each sample at each time point. (C–H) The effect of *edin* RNAi and

overexpression on the production of *Diptericin B* (C), *Cecropin A1* (D), *Attacin B* (E), *Attacin A* (F), *Attacin C* (G) and *Drosocin* (H). n = 4 for each sample at each time point. Error bars represent the standard deviation of each sample.
doi:10.1371/journal.pone.0037153.g005

homozygous Rel^{E20} mutant background using a *C564-GAL4;*Rel^{E20} line. In the homozygous Rel^{E20} background, AMP production via the Imd pathway is eliminated making the flies very sensitive to infections with Gram-negative bacteria [24]. To test whether Edin had antimicrobial properties against Gram-negative or Gram-positive bacteria *in vivo*, we infected the *UAS-edin,*Rel^{E20} flies crossed with the *C564-GAL4;*Rel^{E20} driver with the Gram-positive bacterium *L. monocytogenes* (Figure 7C), which has a DAP-type peptidoglycan, with the Gram-negative bacterium *E. cloacae* (Figure 7D), and with the Gram-positive bacterium *E. faecalis* (Figure 7E). In this homozygous Rel^{E20} background, overexpression of *edin* did not affect the survival rate upon septic injury with any of these microbes. In addition, no rescue was observed after a septic *E. coli* infection (data not shown). According to the results, e*din* overexpression was not sufficient to rescue the flies from succumbing to bacterial infection (Figure 7C–E) indicating that Edin alone does not possess sufficient antimicrobial properties against Gram-negative or Gram-positive bacteria.

To test whether Edin has antimicrobial properties in the context of a normal functioning immune response in *Drosophila*, we overexpressed *edin* in a heterozygous Rel^{E20} mutant background. *Edin* overexpression flies crossed with *C564-GAL4* were infected with *L. monocytogenes* (Figure 7F), *E. cloacae* (Figure 7G) and *E. faecalis* (Figure 7H) and monitored for survival. As shown in Figure 7F–H, overexpressing *edin* did not protect the flies from the bacterial infection. Together these results indicate that Edin has no antimicrobial properties against either Gram negative or Gram positive bacteria *in vitro* or *in vivo*. These results argue that Edin has another immune response modulating function.

Edin is required for normal resistance against bacteria

Next, we investigated whether Edin is required for normal resistance against septic infection. To this end *edin* RNAi flies were crossed with the *C564-GAL4* driver or w^{1118} flies as a control, and the one-week-old offspring were infected with *E. cloacae*, *E faecalis* or *L. monocytogenes*. Rel^{E20} mutant flies were used as a positive control in the *E. cloacae* and *L. monocytogenes* infection model, and *UAS-MyD88* RNAi flies crossed with the *C564-GAL4* driver as a positive control in the *E. faecalis* infection model. When infected with the Gram-negative bacterium *E. cloacae*, Rel^{E20} mutant flies succumbed to the infection within 24 h. *Edin* RNAi flies crossed with *C564-GAL4* flies showed a mild decrease in survival after *E. cloacae* infection compared to *edin* RNAi flies crossed with w^{1118} (Figure 8A) but this it not significant because the *C564-GAL4* driver flies crossed to w^{1118} are more susceptible to the infection. However, a decrease in survival was observed in *edin* RNAi flies infected with the Gram-positive bacterium *E. faecalis* (Figure 8B). However, no statistically significant difference in survival was seen after an *L. monocytogenes* infection (Figure 8C), although a similar trend in survival could be observed, which is in line with the results by Gordon et al. [15]. These results imply that the expression of *edin* might be required for normal resistance against some bacterial infections.

Discussion

In *Drosophila*, the expression of many genes is induced in response to microbial infection. In this study, we examined the role of the infection-inducible gene *edin* in the immune response of *Drosophila melanogaster* both *in vitro* and *in vivo*. We show that *edin* is highly induced in S2 cells by *E. coli* and its expression is dependent on the NF-κB transcription factor Relish both *in vitro* and *in vivo*. In line with the results of Verleyen and coworkers [16], we observe that Edin has a functional signal sequence leading to its cleavage and secretion from S2 cells. Despite the fact that *edin* is highly induced upon infection and that its expression pattern resembles that of known AMPs, we were not able to observe any antimicrobial properties *in vitro* or *in vivo*. Nor were we able to see any bacterial binding or opsonization when these properties of Edin were studied. *Edin* expression also was dispensable for AMP expression via the Imd pathway both *in vitro* and *in vivo*. However, interestingly *edin* RNAi flies showed decreased survival after bacterial infection with *E. faecalis*.

Traditionally, most studies on *Drosophila* AMPs have been successfully carried out *in vitro*. However, *Drosophila* is also a powerful model system for studying the activity of antimicrobial peptides *in vivo*, since it is easy to produce immunocompromised mutant fly lines, which are viable and fertile. Earlier studies have shown that *Drosophila* mutants of the Toll and Imd pathway, that have impaired production of AMPs via these signaling pathways, are highly susceptible to microbial infections [2,4,24] and even a single bacterial cell can be enough to kill a mutant fly [24]. The antimicrobial properties and the microbial specificity of a gene product can be studied by overexpressing the gene of interest in the mutant background of choice. It has been reported that the overexpression of a single antimicrobial peptide in Toll and Imd pathway double mutant flies can restore the resistance to a microbial infection to a level comparable to that of wild-type flies [23]. In our current study, we were not able to demonstrate a broad antimicrobial role for Edin *in vitro* or *in vivo*. *In vitro*, we observed no effect on the colony forming of bacterial cells when Edin was produced in S2 cells or when synthetic peptides were used.

In vivo, the effect of *edin* overexpression on the resistance against microbial infection was analyzed both in a homozygous Rel^{E20} mutant background and in a heterozygous background. Rel^{E20} mutants were selected since they are highly sensitive to Gram-negative bacterial infections. However, no increase in survival after septic injury could be observed in either one of these backgrounds. Therefore it is likely that Edin does not have an antimicrobial role in *Drosophila* although it is highly expressed upon bacterial infection. However, it is also possible that Edin is effective only against a specific microbe which we did not test in our current study. The *in vivo* analysis of antimicrobial properties of a certain peptide is further complicated by the production of a large battery of AMPs that can be partially redundant in their specificities. For instance, Edin alone might not be sufficient to fight against microbial infections, but it may require the presence of another AMP(s), or other immune effector molecules, for full activity.

Previously, Gordon and coworkers [15] have reported that high expression levels of *edin* are detrimental to fly survival and lifespan. We carried out lifespan experiments with our *edin* overexpression fly line and analyzed the proportions of the eclosed progeny. In contrast to Gordon et al., we did not observe a negative effect of *edin* overexpression on fly survival or lifespan. This difference in results could be due to different expression levels of *edin* or different genetic backgrounds of the flies used in these studies. According to our results, the *edin* overexpression fly line used in this study shows expression levels comparable to expression levels upon septic infection (Figure 5B). Furthermore, Gordon et al. [15] reported

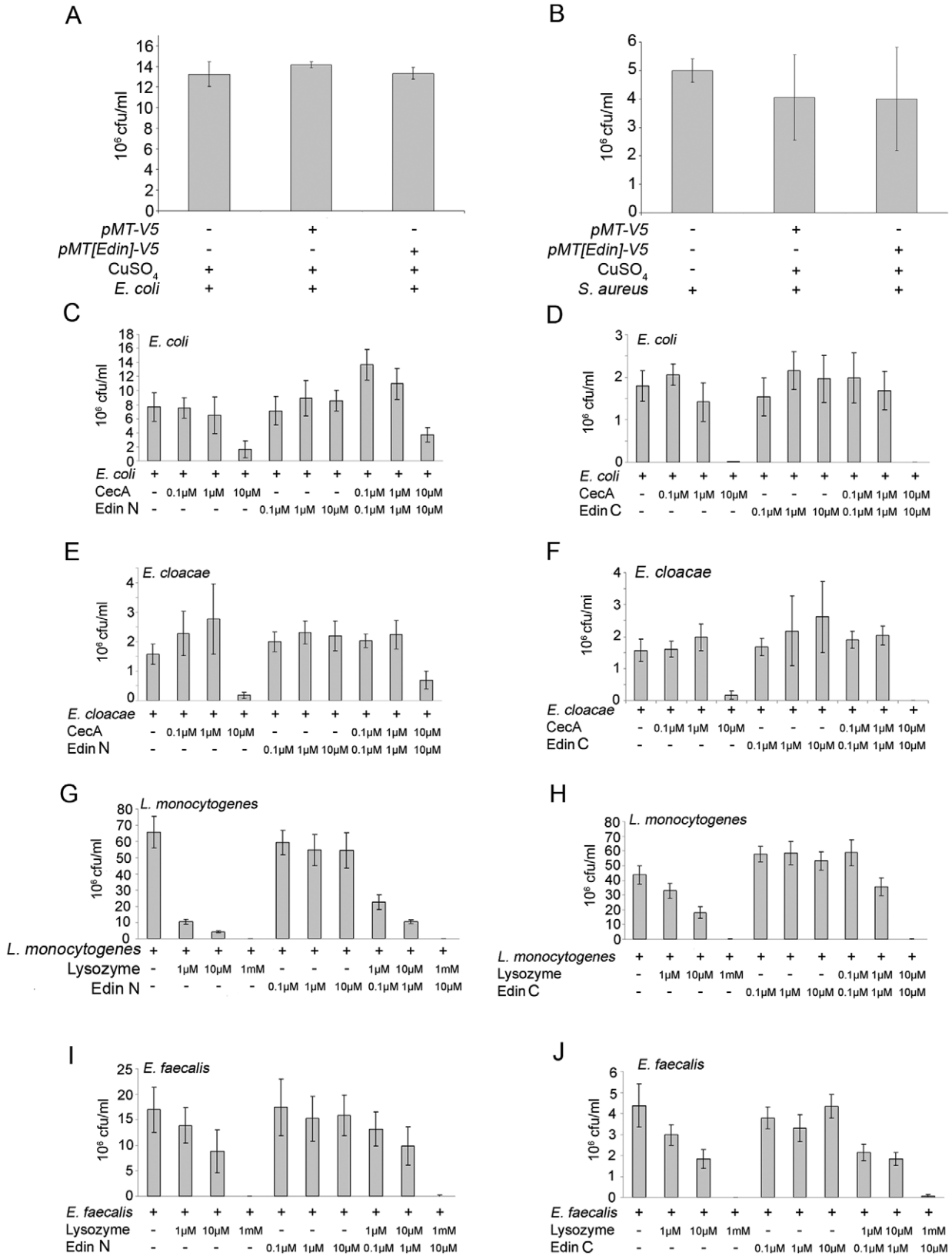

Figure 6. Edin has no broad antimicrobial properties against Gram positive or Gram negative bacteria in vitro. (A–B) Edin does no limit the growth of *E. coli* or *S. aureus* in S2 cell culture medium. S2 cells were transfected with a copper-inducible *pMT-edin-V5* or an empty *pMT* vector, and the abilities of *E. coli* and *S. aureus* to proliferate in these mediums were analyzed. (C–G) Synthetic forms of Edin do not limit the growth of *E. coli* (C), *E. cloacae* (D), *L. monocytogenes* (E), *E. faecalis* (F) or *S. aureus* (G). Both N-terminal and C-terminal forms of Edin were tested. Bacteria were cultured to an $OD_{600\ nm}$ of 0.33, incubated with synthetic Edin and the ability of the bacteria to grow was analyzed. Cecropin A and Lysozyme were used as positive controls for Gram-negative and Gram-positive bacteria, respectively. Left column, N-terminal Edin; right column, C-terminal Edin.
doi:10.1371/journal.pone.0037153.g006

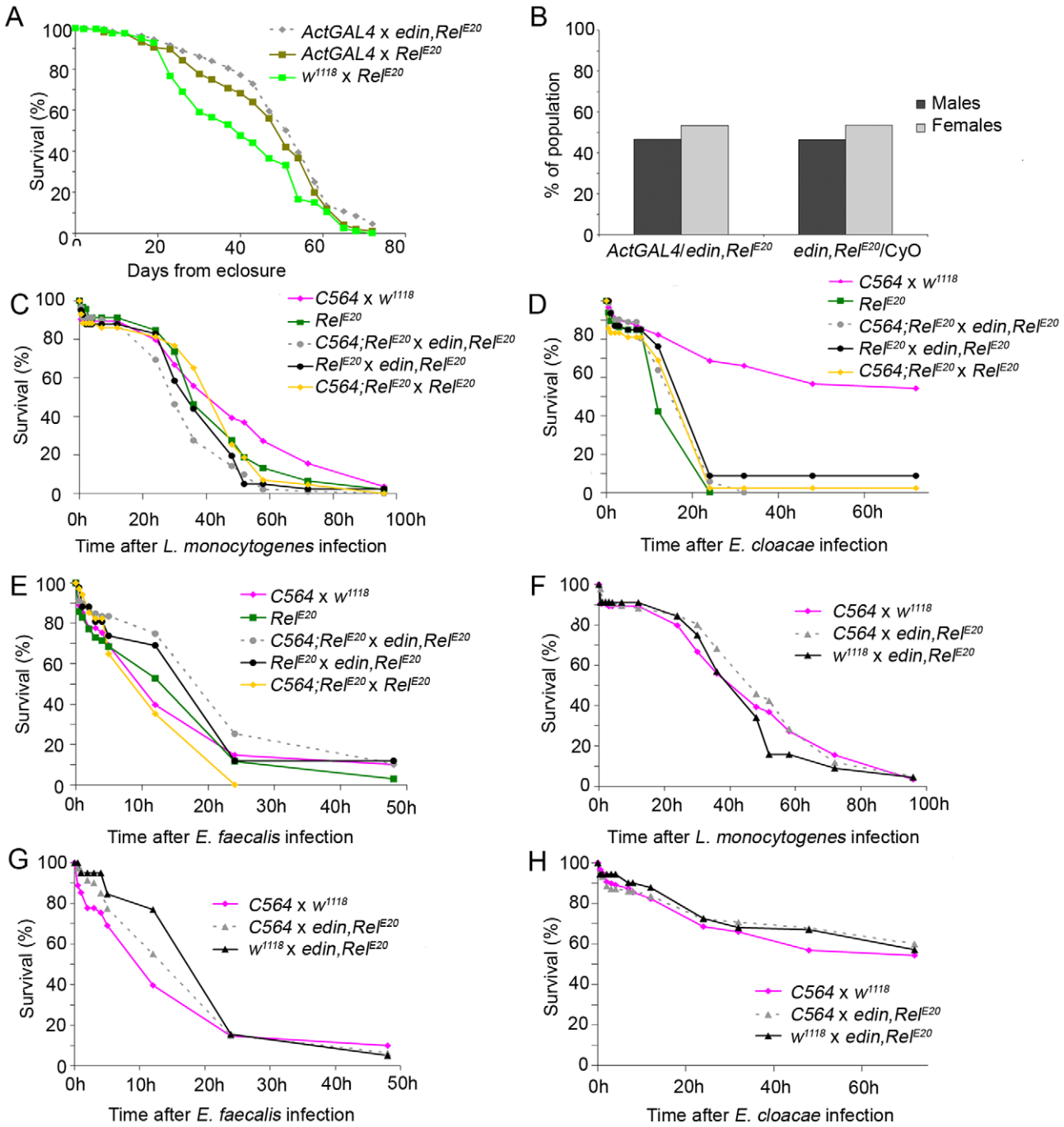

Figure 7. Overexpressing *Edin* has no effect on fly survival after Gram-positive or Gram-negative bacterial challenge in vivo. (A) Overexpressing *edin* does not negatively affect lifespan. *UAS-edin* overexpression flies were crossed with *Actin5C-GAL4* driver lines and the lifespan of their offspring was followed. w^{1118} crossed with Rel^{E20} mutants and *Act5C-GAL4* crossed with Rel^{E20} were used as controls. The data represent one experiment, n = 100 for each cross. (B) Survival is not negatively affected in *UAS-edin* overexpressing flies. Equal amounts of *edin,*Rel^{E20}*/Act5C-GAL4* and *edin,*Rel^{E20}*/CyO* genotypes were obtained from the crosses. (C–H) Flies were pricked with the indicated microbe and survival was followed. (C–E) Overexpressing *edin* in the Rel^{E20} background does not protect the flies from *L. monocytogenes* (C), *E. cloacae* (D) or *E. faecalis* (E) infection. In C–E Rel^{E20} crossed with *edin,*Rel^{E20} and *C564;*Rel^{E20} crossed with Rel^{E20} were used as controls. (F–H) Overexpressing *edin* in a heterozygous w^{1118} background does not protect the flies from *L. monocytogenes* (F), *E. cloacae* (G) or *E. faecalis* (H) infection. *Edin* overexpression flies were pricked with *E. faecalis*, *E. faecalis* or *L. monocytogenes*. *C564-GAL4* flies crossed with w^{1118} and UAS-*edin,*Rel^{E20} crossed with w^{1118} flies were used as controls. Data are pooled from 2–3 experiments which showed similar trends, for each cross (D–J) n = 34–118.
doi:10.1371/journal.pone.0037153.g007

that Edin is required for resistance against *Listeria monocytogenes* infections. *L. monocytogenes* is a DAP-type peptidoglycan containing intracellular bacterium which can infect both mammals and *Drosophila* [25,26]. Gordon et al. [15] report a significant decrease in survival after *L. monocytogenes* infection with two independent *edin* RNAi lines indicating that the normal *edin* expression is required

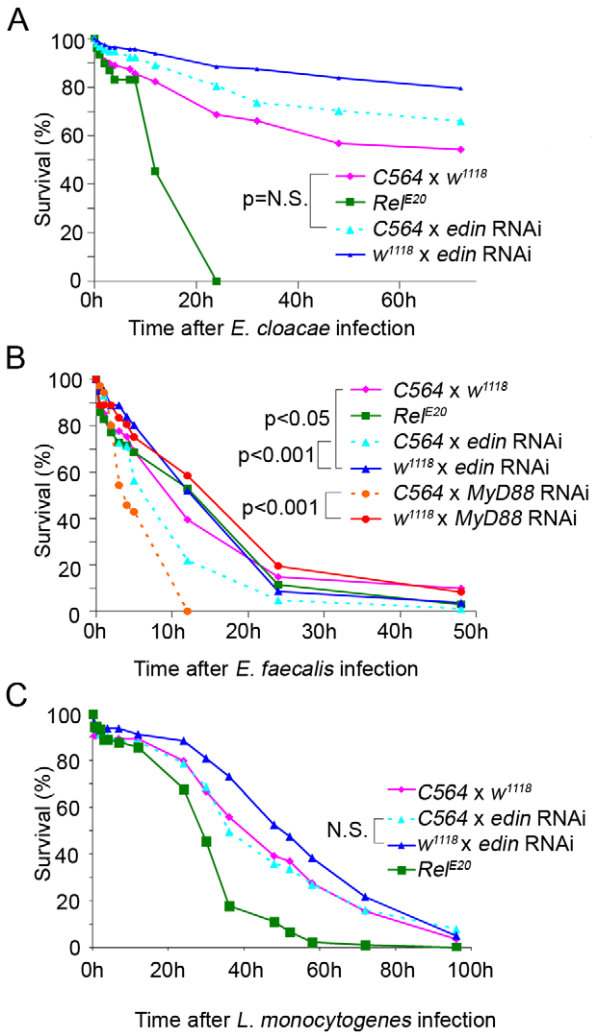

Figure 8. Edin RNAi impairs survival *in vivo* after *E. faecalis* infection. A–C. Healthy adult flies were pricked with a needle dipped either into a culture of *E. cloacae*, *E. faecalis* or *L. monocytogenes* and the survival of the flies was monitored. Rel^{E20} mutants and/or *MyD88* RNAi flies were used as positive controls. (A) Effect of *edin* RNAi after *E. cloacae* infection. Data are pooled from 3 independent experiments which showed similar trends, n = 112–117 for each cross. (B) *Edin* RNAi flies crossed with the *C564-GAL4* driver are more susceptible to *E. faecalis* infection than uninduced *edin* RNAi flies crossed with w^{1118}. Data are pooled from 2 independent experiments which showed similar trends n = 81–87 for each cross. For *MyD88* RNAi crossed to w^{1118} and *C564*, data represents one experiment and n = 35 for both crosses. (C) *Edin* RNAi does not have a significant effect on fly survival against *L. monocytogenes* challenge. Data are pooled from 3 independent experiments which showed similar trends, n = 78–90 for each cross.
doi:10.1371/journal.pone.0037153.g008

for an efficient host response against the pathogen. In our current study, we did not observe a statistically significant reduction in the survival of *edin* RNAi flies after *L. monocytogenes* infection. However, the trend in the survival curve of *edin* RNAi flies is similar to that reported by Gordon et al. Since *Listeria* is an intracellular pathogen, Edin might also have an intracellular function although it is processed and secreted from the cell (Figure 1B). The processed form of Edin is also observed inside the cells (Figure 1B) which would support this hypothesis. However, further studies on the mechanisms involved in resistance against *Listeria* are required to elucidate the role of Edin in the infection.

We also analyzed the role of Edin as a modulator of innate immune signaling cascades. Nevertheless, our experiments indicate that Edin no strong effect on Imd pathway activity either *in vitro* or *in vivo*.

We conclude that the expression of *edin* is Relish-dependent both *in vitro* and *in vivo* but further studies are required to elucidate the exact role of Edin in the immune response in *Drosophila*. Also the mechanisms and signaling pathways involved in the *Listeria monocytogenes* infection remain to be studied.

Materials and Methods

Oligonucleotide microarrays

Oligonucleotide microarray expression data of S2 cells was collected from [14].

Microbial culture

Listeria monocytogenes (strain 10403S), *Enterococcus faecalis*, *Staphylococcus aureus* and *Staphylococcus epidermidis* were cultured in BHI. *Enterobacter cloacae* (strain β12) and *Micrococcus luteus* were cultured in LB supplemented with either 15 ng/ml of nalidixic acid (Sigma-Aldrich, St. Louis, Missouri, USA) or 100 µg/ml of streptomycin (Sigma-Aldrich), respectively. *Serratia marcescens* (strain Db11) and *Escherichia coli* were cultured in LB supplemented with 100 µg/ml of ampicillin. The baker's yeast *Saccharomyces cerevisiae* (AH109) was grown overnight in YPDA medium (Gibco/Life Technologies, Carlsbad, CA, USA) supplemented with 15 µg/ml of kanamycin at +30°C with shaking.

Semi-quantitative and quantitative RT-PCR

Semi-quantitative RT-PCR reactions for *edin*, *Attacin A and Act5C* were performed using Super-Script™ II One-Step RT-PCR with Platinum Taq kit (Invitrogen/Life Technologies, Carlsbad, CA, USA). The following primers were used: *Edin*: 5′-GTTCTCCAACAAGTGCGG-3′ (forward), and 5′- CAGAAATGCCAGGTGCCC-3′ (reverse); *Attacin A*: 5′-TTTGGCCTACAACAATGCTG-3′ (forward), and 5′-GCTTCTGGTTGGCAAACG-3′ (reverse); *Act5C:* 5′-CGAAGAAGTTGCTGCTCTGG-3′ (forward), and 5′-AGAACGATACCGGTGGTACG-3′ (reverse).

Quantitative RT-PCR was carried out using the QuantiTect SYBR Green RT-PCR kit (Qiagen) and an ABI7000 (Applied Biosystems) instrument according to the manufacturer's instructions. Results were analyzed with the ABI 7000 System SDS software version 1.2.3. The following primers were used: *AttB*, 5′-CAGTTCCCACAACAGGACC-3′ (forward) and 5′-CTCCTGCTGGAAGACATCC-3′ (reverse); *Drosocin*, 5′-TTCCTGCTGCTTGCTTGCG-3′ (forward) and 5′-TGGCAGCTTGAGTCAGGTG-3′ (reverse); *AttA*, 5′-GCATCCTAATCGTGGCC-3′ (forward) and 5′-GCTTCTGGTTGGCAAACG-3′ (reverse); *AttC*, 5′-CATCGTTGGCGTACTTGGC-3′ (forward) and 5′-TTGCTGGAAGCTATCCCGC-3′ (reverse); *CecA1*, 5′-CGTCGCTCTCATTCTGGC-3′ (forward) and 5′-GTTGCGGCGACATTGGC-3′ (reverse); *DptB*, 5′-GACTGGCTTGTGCCTTC-3′, and 5′-CCTGAAGGTATACACTCC-3′ (reverse); and *Edin*, 5′-CTCGTGTCCTGCTGTCTG-3′ (forward), and 5′-GCCTTCGTAGTTGTTCCG-3′(reverse).

S2 cell culture and transfections

Drosophila hemocyte-like S2 cells [27] (obtained from Invitrogen/Life Technologies) were maintained in Schneider's Insect Cell Culture Medium (Sigma-Aldrich, St. Louis, Missouri, USA) supplemented with 10% FBS, 100 U/ml Penicillin and 100 µg/ml Streptomycin (Sigma-Aldrich) at +25°C. The cells were transfected using the Fugene® transfection reagent (Roche Applied

Science, Penzberg, Germany) according to the manufacturer's instructions.

Cloning and constructs

Edin was cloned into the pMT/V5-HisA (Invitrogen/Life Technologies) and pUAST [28] vectors using S2 cell cDNA as a template. The primers used were 5′-CAGAATTCATGTTCTC-CAACAAGTGC-3′ and 5′-CAGGTACCTCAGAAATGCCA-GGTGCC-3′ for pUAST, and 5′-CAGCGGCCGCATG-TTCTCCAACAAGTGC-3′ and 5′-CACTCGAGGAAATGC-CAGGTGCCCCG-3′ for pMT/V5-His.

Western blotting

S2 cells were transfected with 0.5 µg of pMT[*edin*]-V5. Cells were harvested, pelleted and lyzed 24 h after addition of $CuSO_4$. 25 µg of cell lysate and supernatant were electrophoresed in NuPAGE 12% Bis-Tris gel (Invitrogen Life Technologies), blotted on a nitrocellulose membrane, and detected by Western blotting using mouse anti-V5 primary Ab (Invitrogen/Life Technologies) and goat anti-mouse Ab HRP conjugates (Molecular Probes) together with ECL Plus Western blotting detection system (GE Healthcare Life Sciences, Uppsala, Sweden).

Synthetic peptides

Two forms of synthetic Edin were ordered from Peptide 2.0. (Chantilly, VA, USA). Amino acid sequences: N-terminal form, SYRQ PYPEEF QTSPE QLLQ VAPLV; C-terminal form, SPEGG SVVVT ASKDNQ VGREAS VQYNHN LYSSG DGRGS IDAYA QASRN FDYNR NNYEG GIRGT WHF. The peptides were dissolved in H_2O according to the manufacturer's instructions.

Colony forming unit assay

Edin-V5 expressed in S2 cells: S2 cells in 48-well plates in an antibiotic-free medium were transfected with 0.5 µg of pMT-*edin*-V5 plasmid or an empty plasmid. Expression of the plasmid was induced 48 h later by adding $CuSO_4$ to a final concentration of 300 µM. 100 µl of overnight grown bacterial suspension ($OD_{600\ nm} = 0.33$, $\sim 1*10^6$ bacteria/ml) was centrifuged and resuspended in 1 ml of Schneider medium supplemented with 10% FBS. 50 µl of *E. coli* and *S. aureus* suspension were added to the wells 24 h after $CuSO_4$ and incubated for 2 h at +25°C. Serial dilutions of the bacterial suspensions were made in sterile water. 20 µl droplets of each dilution were pipetted on LB (*E. coli*) or BHI (*S. aureus*) agar plates, the plates incubated overnight at +37°C and the bacterial colonies counted.

Synthetic forms of Edin: An overnight grown bacterial suspension ($\sim 1*10^6$ bacteria/ml) was centrifuged and washed as above and resuspended in 5% DMSO. 5 µl of *E. coli*, *E. cloacae*, *L. monocytogenes* and *E. faecalis* suspension were added on the 96-well plates containing synthetic Edin at concentrations of 10 µM, 1 µM and 100 nM. Suspensions were incubated for 2 h at +25°C, after which serial dilutions were made in sterile water. Dilutions were plated as above and the bacterial colonies counted. Lysozyme and Cecropin A (Sigma-Aldrich, St. Louis, Missouri, USA) were used as a positive control for Gram-positive and Gram-negative bacteria, respectively.

Luciferase reporter assays and dsRNA treatments

Luciferase reporter assays to analyze the Imd, Toll and JAK/STAT pathways, and dsRNA treatments were carried out as described earlier [29,30].

Drosophila stocks

The *edin* RNAi line (stock #14289) was obtained from VDRC and the *C564-GAL4* flies were a kind gift from from Prof. Bruno Lemaitre (Global Health Institute, EPFL, Switzerland). *CG32185* transgenic flies were generated by microinjecting the *pUAST-edin* construct to the Rel^{E20} background in the Umeå Fly and Worm Transgene Facility. The genotype of the *edin* overexpression fly line is w;+;*UAS-edin*,Rel^{E20}.

Lifespan experiments

UAS-edin flies were crossed with *C564-GAL4*, *Actin5C-GAL4/CyO* and *Daughterless-GAL4* driver flies. Rel^{E20} crossed with driver flies were used as a control. The lifespan of the offspring of the crosses was monitored at +25°C. Flies were moved to vials containing 5 ml of fresh fly food twice a week and their survival was monitored. Males and females were kept in separate vials, 10 to 20 flies per vial.

Infection experiments

Infections were carried out by pricking one week-old healthy flies with a thin tungsten needle dipped in a concentrated pellet of either *Escherichia coli*, *Enterobacter cloacae* (strain β12), *Enterococcus faecalis* or *Listeria monocytogenes* (strain 10403S) which were grown overnight on culture plates.

RNA extraction from flies

Quadruplicates of five flies (2 females and 3 males) were snap frozen on dry ice 0 h, 1 h, 4 h or 8 h post-infection. Flies were homogenized in TRIsure reagent (Bioline, London, UK) and total RNAs were extracted according to the manufacturer's instructions.

Statistical analysis

Statistical analyses of results were carried out using one-way ANOVA. For survival experiments, Log Rank analysis was carried out and $p<0.05$ was considered to be significant.

Flow cytometry

The amount of cell-associated microbes was analyzed using flow cytometry as described earlier [20].

Binding assay

The binding assay for Edin was carried out essentially as described earlier [20] with minor modifications. In brief, S2 cells were seeded onto 24-well plates and transfected with 0.5 µg of pMT-*edin*-V5 plasmid or an empty pMT-V5 plasmid. $CuSO_4$ was added 48 h later to a final concentration of 500 µM. Cells were harvested the next day and the supernatant was collected. 1 ml of overnight grown microbial culture was centrifuged and the pellet was washed 5 times with 1× PBS. 500 µl of medium containing either pMT-*edin*-V5 or empty pMT-V5 was added in the tubes containing the microbial pellets or latex beads treated with 0.4 M N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide (EDC) and 0.1 M N-hydroxysuccinimide (NHS) and coated with BSA (10 mg/ml in PBS, pH 7.4). The samples were incubated for 1 h in an end-to-end rotator at +4°C. Thereafter the samples were washed five times with 1 ml of 1× PBS and the pellet was suspended in 20 µl of PBS. To detach the bound Edin from the microbial cells, SDS-PAGE sample buffer was added and the suspension was boiled for 10 min. The samples were centrifuged and 30 µl of the supernatant was loaded on to a 12% NuPAGE BisTris gel, electrophoresed and the proteins were transferred to a nitrocellulose membrane as described above.

Acknowledgments

We thank other members of our laboratory, Prof. Shoichiro Kurata for the *L. monocytogenes* strain and VDRC for the *edin* RNAi line. We are also grateful to Ingrid Dacklin in Umeå Fly and Worm Transgene Facility for performing microinjections to fly embryos. The fly work was done at University of Tampere *Drosophila* Core Facility funded by Biocenter Finland. All authors approved the final version of the text.

Author Contributions

Conceived and designed the experiments: LMV AK JU DH SV MR. Performed the experiments: LMV AK MK JU BW DH. Analyzed the data: LMV AK MK JU DH SV MR. Wrote the paper: LMV AK JU BW DH SV MR.

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Edin Expression in the Fat Body Is Required in the Defense Against Parasitic Wasps in *Drosophila melanogaster*

Leena-Maija Vanha-aho[1], Ines Anderl[2,3], Laura Vesala[2], Dan Hultmark[2,3], Susanna Valanne[1], Mika Rämet[1,4,5*]

[1] Laboratory of Experimental Immunology, BioMediTech, University of Tampere, Finland
[2] Laboratory of Genetic Immunology, BioMediTech, University of Tampere, Finland
[3] Department of Molecular Biology, Umeå University, Umeå, Sweden
[4] Department of Pediatrics, Tampere University Hospital, Tampere, Finland
[5] Department of Pediatrics, Medical Research Center Oulu, University of Oulu and Department of Children and Adolescents, Oulu University Hospital, Oulu, Finland

Abstract

The cellular immune response against parasitoid wasps in *Drosophila* involves the activation, mobilization, proliferation and differentiation of different blood cell types. Here, we have assessed the role of Edin (elevated during infection) in the immune response against the parasitoid wasp *Leptopilina boulardi* in *Drosophila melanogaster* larvae. The expression of *edin* was induced within hours after a wasp infection in larval fat bodies. Using tissue-specific RNAi, we show that Edin is an important determinant of the encapsulation response. Although *edin* expression in the fat body was required for the larvae to mount a normal encapsulation response, it was dispensable in hemocytes. *Edin* expression in the fat body was not required for lamellocyte differentiation but it was needed for the increase in plasmatocyte numbers and for the release of sessile hemocytes into the hemolymph. We conclude that *edin* expression in the fat body affects the outcome of a wasp infection by regulating the increase of plasmatocyte numbers and the mobilization of sessile hemocytes in *Drosophila* larvae.

Author summary

The events leading to a successful granuloma formation to seal objects that are too large to be eliminated, are insufficiently understood. The encapsulation of parasitoid wasp eggs in the larvae of the fruit fly *Drosophila melanogaster* is an example of a similar process in invertebrates. Like granuloma formation in Mycobacterial infection in humans, the encapsulation process requires the activation, mobilization, proliferation and differentiation of different blood cell types. Here, we have studied the role of Edin (elevated during infection) in the immune defense against the parasitoid wasp *Leptopilina boulardi* in *Drosophila* larvae. We demonstrate that Edin expression in the fat body (an immune-responsive organ in *Drosophila* functionally resembling the mammalian liver) is required for a normal defense against wasp eggs. Edin is required for the release of blood cells from larval tissues and for the subsequent increase in circulating blood cell numbers. Our results provide new knowledge of how the encapsulation process is regulated in *Drosophila*, and how blood cells are activated upon wasp parasitism. Understanding of the encapsulation process in invertebrates may eventually lead to a better knowledge of the pathophysiology of granuloma formation in human diseases, such as tuberculosis.

Introduction

Parasitoid wasps are natural enemies of insects such as the fruit fly *Drosophila melanogaster*. In the course of a wasp infection, a female wasp lays an egg in a fruit fly larva and the wasp larva hatches. Thereafter, the wasp larva develops inside the *Drosophila* larva using the host tissue as a source of nutrition to ultimately emerge as an adult wasp, unless the wasp larva is eliminated by the host's immune response [1].

The initial oviposition of a wasp egg triggers changes in gene expression in the fruit fly and activates both humoral and cellular defense mechanisms [2-4]. The role of the humoral defense, i.e. the production of antimicrobial peptides (AMPs) by the fat body, via the Imd and Toll pathways in response to a microbial challenge, is well characterized in response to microbial challenge (reviewed in [5, 6]). However, in the context of wasp parasitism, cellular immunity is more striking than the humoral response. The cellular immune responses are mediated by three types of blood cells, or hemocytes: plasmatocytes, lamellocytes and crystal cells (reviewed for example in [7, 8]). The round and small plasmatocytes are the most abundant type tallying up to 95% of all of the larval hemocytes. Plasmatocytes are responsible for phagocytosing invading microorganisms and apoptotic particles and are also required for a normal resistance against bacteria [9-12]. Crystal cells comprise around 5% of all hemocytes and they contain phenoloxidase-containing crystals that are released in the melanization response [13]. Lamellocytes, on the other hand, are solely found in larvae and are rarely present in individuals that are not immune-challenged. The main task of lamellocytes is to participate in encapsulating objects that are too large to be phagocytosed, such as the eggs of parasitoids wasps. However, the encapsulation of wasp eggs requires the concerted action of all three types of hemocytes [7].

Upon a wasp infection, the presence of a wasp egg is first recognized. This then leads to the differentiation of a large number of lamellocytes [14-16], which migrate towards the wasp egg and attach to it. During a successful immune response lamellocytes, together with plasmatocytes, form a multilayered capsule that surrounds the wasp egg. The capsule is melanized, phenol oxidases and reactive oxygen species are released within the capsule [17], and the wasp is ultimately killed.

Although many pathways, such as the Toll and JAK/STAT pathway, have been shown to have a role in the encapsulation response [3], the phenomenon is still insufficiently understood. In this current study, we investigate the role of Edin (elevated during infection) in a wasp infection. Edin is a small peptide that is secreted into the hemolymph upon infection [18, 19], and it is required for the immune response against *Listeria monocytogenes* [20]. Earlier, we have shown that the expression of *edin* is induced after a bacterial infection, and it has a minor role in the resistance against *Enterococcus faecalis* [19]. In this study, we investigated whether *edin* expression is induced by a wasp infection using the *Leptopilina boulardi* strain G486. We also examined the role of Edin in the encapsulation response and in the activation and formation of hemocytes upon a wasp infection. We report that *edin* expression is required in the fat body upon a wasp infection in order to mount an effective encapsulation response, and that knocking down *edin* in the fat body causes an aberrant hemocyte phenotype in *Drosophila* larvae.

Results

Edin is induced upon a wasp infection

We have previously shown that *edin* is induced both *in vitro* and *in vivo* upon a microbial infection, but were unable to find any essential role for Edin in this context [19]. To test whether a wasp infection induces the expression of *edin*, we infected *Canton S* larvae with the parasitoid wasp *Leptopilina boulardi* strain G486, and determined the expression levels of *edin* in whole larvae three hours after infection using qRT-PCR. As is seen in Figure 1A, the wasp infection led to a 7-fold induction in the expression levels of *edin* compared to uninfected larvae. Because the fat body is the main immune-responsive organ in the fruit fly, we next looked at *edin* mRNA levels in the fat bodies of wasp-infected larvae 24 hours post-infection. As is shown in Figure 1B, the expression of *edin* was more highly induced in the fat bodies of the wasp-infected larvae than in whole larvae (80-fold induction). Our results indicate that *edin* is upregulated after a wasp infection in larvae and that the fat body is an important organ for its expression.

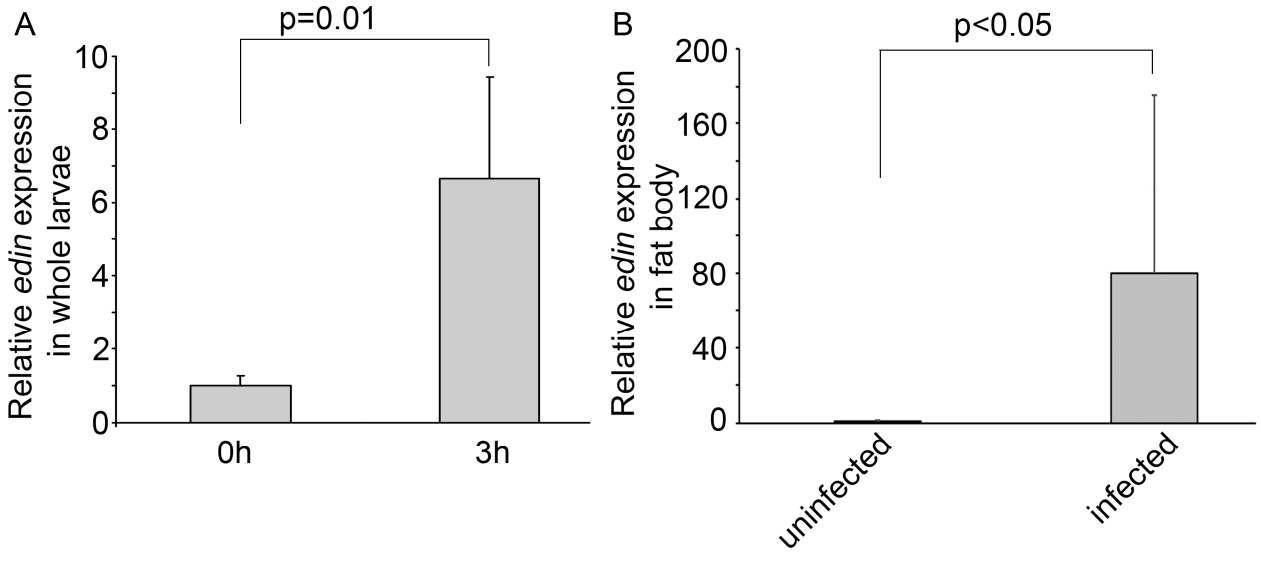

Figure 1. *Edin* expression is induced upon a wasp infection. (A) A wasp infection causes a 6.7-fold increase in *edin* expression in 2nd instar *Canton S* larvae. Data are pooled from two independent experiments, n=2 for each experiment, where one sample represents 10 larvae. **(B)** *Edin* expression is induced in the fat bodies of *Canton S* larvae 24 hours post infection. The data are pooled from four independent experiments, n=2 for each experiment, one sample representing 8-10 larval fat bodies.

Edin expression in the fat body is required for the normal encapsulation of wasp eggs

Fruit fly larvae can mount an effective immune response against invading parasitoids by encapsulating the wasp egg. To address the functional significance of *edin* expression for the encapsulation process upon an *L. boulardi* infection, we used the UAS-GAL4 system to knock down *edin* expression. The normal response against the wasp egg is the formation of a visible melanized capsule around the parasitoid egg, and in our hands, 45-66% of control larvae showed a melanized capsule. First, we crossed $edin^{109528}$ RNAi flies (#109528, hereafter referred to as $edin^{109528}$) with the *C564-GAL4* driver line and looked for the presence of melanized capsules 27-29 hours after the wasp parasitization (Figure 2A). Parasitized w^{1118} controls showed an encapsulation rate of 47%. Similarly, w^{1118} crossed with *C564-GAL4* or $edin^{109528}$ showed encapsulation rates of 51% and 45%, respectively, while only 6% of $edin^{109528}$crossed with *C564-GAL4* showed melanized capsules. To ensure that the observed phenotype was caused by reduced *edin* expression, we analyzed the encapsulation response of another *edin* RNAi line (#14289, hereafter referred to as $edin^{14289}$). Similarly to the $edin^{109528}$ line, $edin^{14289}$crossed with the driver line showed a clearly decreased encapsulation efficiency of 14% (Figure 2A), when compared to $edin^{14289}$ crossed with w^{1118}.

The *C564-GAL4* driver is expressed in many organs, including the fat body, salivary glands and lymph glands [21]. We next used a fat body-specific driver to examine specifically whether the lowered encapsulation response was due to the role of *edin* in the fat body. We crossed both the $edin^{109528}$ and $edin^{14289}$ RNAi lines with the *Fb-GAL4* driver line and examined the encapsulation response of the offspring. *Fb-GAL4* crossed with w^{1118} showed encapsulation levels of 45% (Figure 2A), whereas *edin RNAi* flies crossed with *Fb-GAL4* showed an encapsulation activity of only 8% ($edin^{109528}$) and 7% ($edin^{14289}$). In addition, similar results were also obtained with another fat body-specific driver, *Lsp2-GAL4* (data not shown).

We also analyzed the encapsulation activity of *edin* RNAi larvae crossed with the pan-hemocyte driver *Hml*$^{\Delta}$>GFP;*He*>GFP and were not able to see any effect with either of the RNAi lines (Figure 2A). Together, these data suggest that Edin is required for a normal encapsulation response after parasitization, and that its expression is required in the larval fat body but not in the hemocytes.

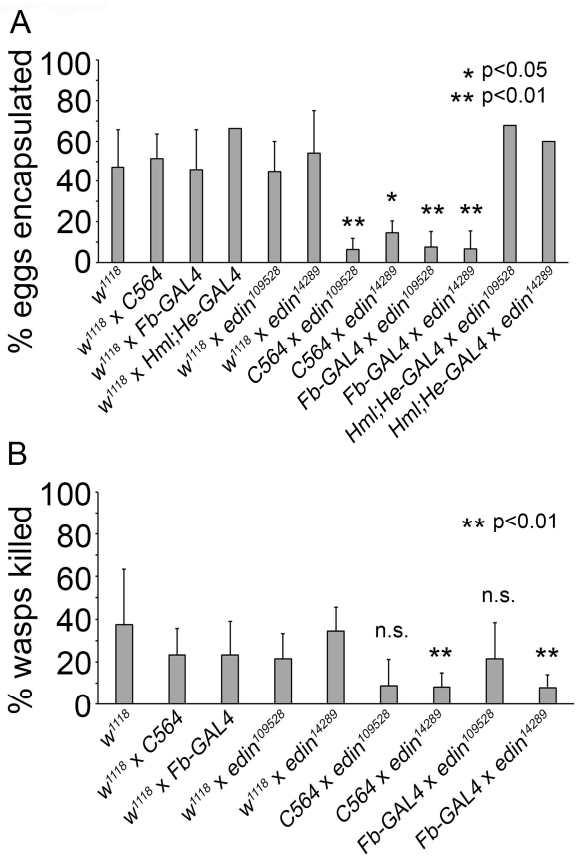

Figure 2. Knock down of *edin* in the fat body decreases the encapsulation and killing ability of *Drosophila* larvae. (A) The encapsulation response of two different *edin* RNAi (VDRC #14289 and #109528) lines was analyzed 27-29h after a wasp infection. The *C564-GAL4, Fb-GAL4* and *Hml*$^{\Delta}$*;He-GAL4* drivers were used to drive the expression of the RNAi constructs. Knocking down the expression of *edin* in several tissues including the fat body or in the fat body alone caused a significant decrease in the encapsulation activity compared to controls. Knocking down *edin* in the hemocytes had no effect on the encapsulation activity of fruit fly larvae. Wasp eggs were counted as encapsulated when they were melanized. Data are pooled from three to six independent experiments, with the exception of *Hml*$^{\Delta}$*;He-GAL4* x *w*^{1118}, *Hml*$^{\Delta}$*;He-GAL4 x edin*^{109528} and *Hml*$^{\Delta}$*;He-GAL4* x *edin*^{14289}, which represent one experiment, and 100 larvae per column. Otherwise, at least 50 infected larvae were scored per sample per experiment. **(B)** *Edin* RNAi lines (VDRC #14289 and #109528) were infected with wasps and the ability of the larvae to kill the wasp egg was analyzed 48-50h after infection. Wasp eggs were counted as dead, if they were melanized and no wasp larva had hatched. The *C564-GAL4* and *Fb-GAL4* drivers were used to drive the expression of the RNAi constructs. Data are pooled from at least three independent experiments, and at least 50 infected larvae were scored per sample per experiment.

Edin expression is required for the resistance against wasp parasitism in *Drosophila* larvae

Scoring for the ability of the fly larva to melanize the wasp egg does not indicate whether the fruit fly larva is actually able to overcome the parasitization. Therefore, we replicated the experimental setting in Figure 2A, but scored for the presence of living or dead wasp larvae 48-50 hours post infection. The parasite was scored as killed by the fruit fly larva if a melanized wasp egg was found in the hemocoel in the absence of a living wasp larva. As is seen in Figure 2B, the percentage of dead wasp larvae is reduced compared to controls when *edin* RNAi is induced ($edin^{109528}$ and $edin^{14289}$) with either the *C564-GAL4* or *Fb-Gal4* driver, although with the combination of the $edin^{109528}$ RNAi line and the *C564-GAL4* driver the resulting suppression was not statistically significant (9% compared to 22-38 % in controls). Crossing the $edin^{14289}$ RNAi line with both the *C564-GAL4* and *Fb-GAL4* driver resulted in a killing rate of only 8% ($p<0.01$ compared to w^{1118} crossed with $edin^{14289}$). No significant effect, however, was seen when $edin^{109528}$ was crossed with *Fb-GAL4*, but the proportion of killed wasps stayed at 21% (Figure 2B). These results, together with the encapsulation phenotype, indicate that Edin is required for the resistance against wasp parasitism in *Drosophila* larvae.

Edin expression is not required for lamellocyte differentiation in *Drosophila* larvae upon *L. boulardi* parasitism

Lamellocytes have a central role in the resistance against *L. boulardi* parasitism. They are not found in the hemocoel of healthy, unchallenged *Drosophila* larvae, but they are formed in response to a wasp infection [15, 16, 22]. To investigate whether the expression of *edin* is required for lamellocyte formation, we visualized the hemocytes of wasp-challenged larvae 48-50 hours after infection. Plasmatocytes and lamellocytes were visualized using the *eaterGFP* (green) and *msnCherry* (red) reporters, respectively. The hemocytes were bled onto microscope slides and visualized under a fluorescence microscope. As is shown in Figures 3A and 3B, most of the hemocytes in the unchallenged larvae express the *eaterGFP* reporter and are *msnCherry*-negative, indicating that only

plasmatocytes are present. Lamellocytes are *msnCherry*-positive, large, and flat cells. They are present only in the infected larvae (Figure 3A' and 3B') and are found both in RNAi treated and control larvae, indicating that *edin* expression in the fat body is not required for lamellocyte formation upon a wasp infection (Figure 3B'). It is noteworthy that the infected larvae contain cells that express both *eaterGFP* and *msnCherry* reporters, showing that some of the cells are undergoing plasmatocyte to lamellocyte transition and are not yet fully differentiated lamellocytes (Figure 3A' and Supplementary Figure 1).

In order to obtain additional information about the role of Edin in hemocyte differentiation upon a wasp infection, we analyzed hemocytes of *edin* knock-down larvae with flow cytometry utilizing the *eaterGFP,msnCherry* reporter. Figures 3C-D' show representative scatter plots of hemocytes of uninfected and infected larvae with *edin* RNAi in the fat body as well as age-matched uninfected and infected control larvae at the 27-29 hour time point. Lamellocytes were induced in spite of *edin* depletion in the fat body. When comparing hemocyte numbers of uninfected and infected control larvae and *edin* RNAi larvae, we found that although lamellocyte numbers of infected animals did not change, the plasmatocyte numbers generally increased approximately two to three fold after infection but remained constant in *edin* knock-down larvae (Figure 3E). Taken together, Edin was dispensable for lamellocyte formation but seemed to be necessary to increase plasmatocyte numbers after a wasp infection.

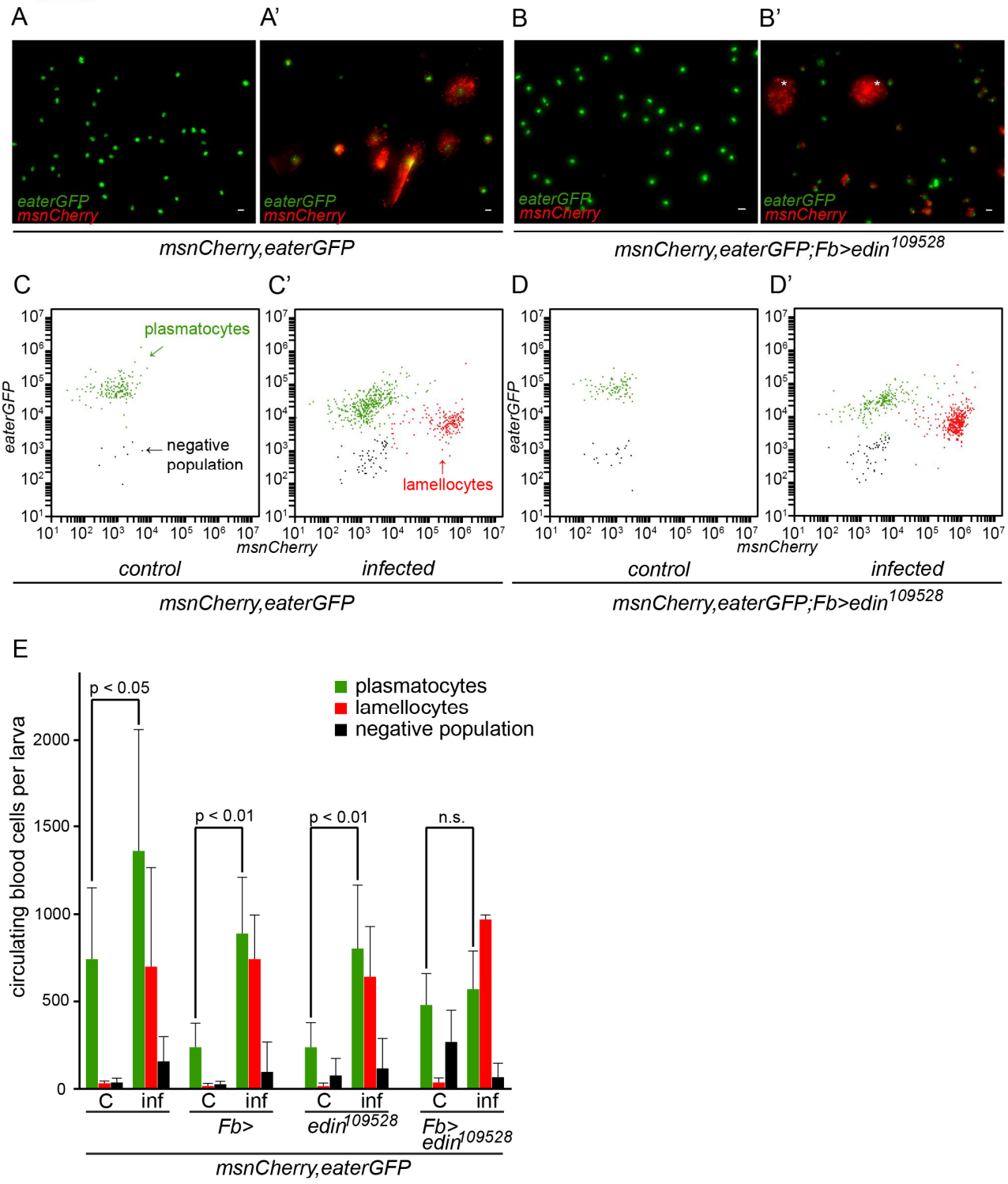

Figure 3. Quantification of hemocytes in *edin* RNAi larvae after a wasp infection. (A-B) Hemocytes of infected larvae were bled 48-50 hours post-infection and visualized with the *eaterGFP* (green) and *msnCherry* (red) reporters. Uninfected controls contained only GFP-positive cells that corresponded to plasmatocytes (green). (**A' and B'**) *msnCherry* expression was detected in the infected samples and this included lamellocytes (asterisks) and cells that express both *eaterGFP* and *msnCherry* indicating that they were

undergoing lamellocyte transition. Lamellocytes were present also in the infected *edin* RNAi larvae suggesting that *edin* expression is not necessary for lamellocyte differentiation. Scale bars are 10 μm **(C-E)** Flow cytometry was carried out to quantify the amount of hemocytes in the unchallenged and the wasp infected *edin* RNAi larvae. (C = control, inf = infected)

Knocking down *edin* in the fat body causes an altered hemocyte phenotype in wasp-infected larvae

To look at phenotypic differences that could not be studied with flow cytometry, such as the localization of hemocytes, we next visualized wasp-infected larvae using fluorescence microscopy. We imaged the *Fb-GAL4* driven *edin* RNAi larvae and the respective control crosses 27-29 hours after the wasp parasitization, and again used the *eaterGFP,msnCherry* reporter line to allow the visualization of plasmatocytes (green) and lamellocytes (red). In the uninfected controls (Figure 4A-D, top row), the banded pattern of plasmatocytes and the lymph gland could been seen. The bands represented plasmatocytes that resided in the sessile compartment in the absence of an immune stimulus. When the larvae were infected by wasps, the green banded pattern disappeared (Figure 4E-G) and lamellocytes appeared in the hemolymph (Figure 4E'-G'). This was due to the activation of the hemocytes in the sessile compartment in response to the wasp infection, which causes the cells to leave the compartment and enter the circulation, where many differentiate into lamellocytes [23, 24]. Consistent with our flow cytometry data, when *edin* was knocked down in the fat body, lamellocytes still appeared in the circulation showing that Edin did not affect formation of lamellocytes (Figure 4H'). However, in the *edin* knockdown larvae the banded pattern of plasmatocytes was not disrupted as in the controls (Figures 4H and 4H'). Of note, overexpression of *edin* in the fat body did not disrupt the banded pattern indicating that the overexpression of *edin* alone was not sufficient for releasing the sessile hemocytes into the circulation (data not shown). In conclusion, our data suggest that *edin* expression in the fat body affects plasmatocyte activation and release from the sessile compartment,

meaning that the silencing of *edin* results in a compromised response to *L. boulardi* parasitism in the early stages (27-29 hours) of the infection.

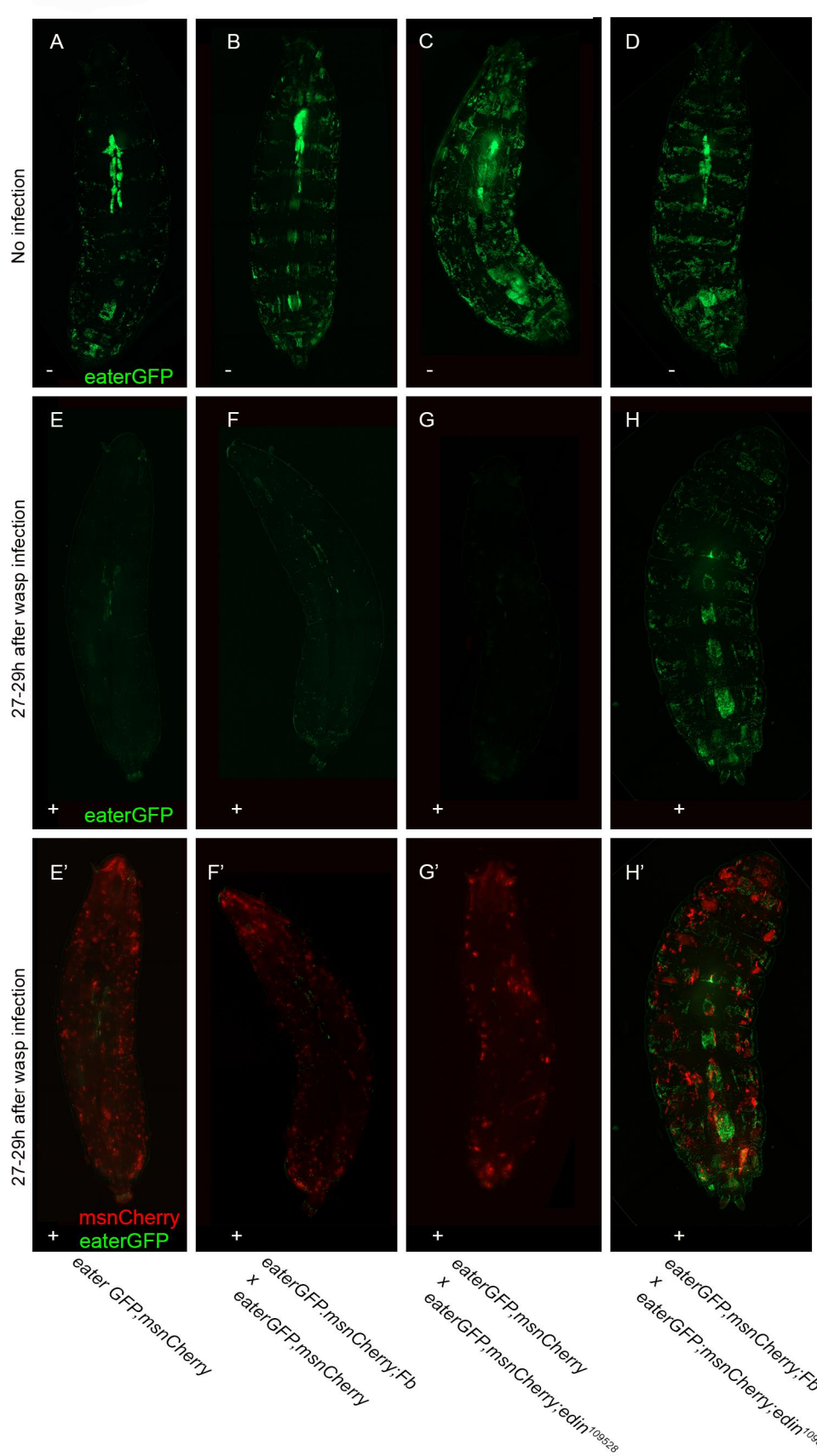

Figure 4. *Edin* expression in the fat body is required for the activation of plasmatocytes upon a wasp attack 27-29 hours after infection. The *in vivo* phenotype of wasp infected *edin* RNAi larvae was studied using the *eaterGFP* (green=plasmatocytes) and *mCherry* (red=lamellocytes) reporters. Imaging was performed 27-29 hours post infection with living *Drosophila* larvae. **(A-D)** Uninfected larvae show an uninterrupted banding pattern formed by sessile plasmatocytes (green). **(E-H)** Shows only the green channel (eaterGFP) of infected larvae and **(E'-H')** both the green and the red (msnCherry) channel. Infected larvae have lost the banding pattern and lamellocytes have appeared, but infected *eaterGFP,msnCherry;Fb-GAL4 x eaterGFP,msnCherry;Edin RNAi* larvae still show a visible banding pattern formed by the sessile cells. – = uninfected larvae, + = wasp infected larvae. Figure 4 shows representative images of at least 10 larvae per condition and per genotype were imaged.

Discussion

Encapsulation is an elegant yet complex response against a wasp attack in fruit fly larvae and it requires the concerted action of activated hemocytes. In addition to inducing the encapsulation response, a wasp infection causes changes in the expression profile of the fruit fly genes [3, 4]. Our results show that the immune-inducible gene *edin* was rapidly induced in response to an infection by the endoparasitoid wasp *Leptopilina boulardi* and that *edin* expression in the fat body, but not in hemocytes, was required to mount a normal encapsulation response against the wasp. Encapsulation was not blocked entirely, however. When *edin* was knocked down in the fat body, only approximately 10% of the larvae encapsulated the wasp egg normally. In addition to the encapsulation of the wasp egg, Edin was required to increase plasmatocyte numbers, to efficiently kill the invading wasp larva, and to activate the hemocytes of the sessile compartment upon a wasp infection.

The key players involved in the encapsulation response are the lamellocytes that appear in the circulation after a wasp infection [14, 25]. However, the presence of lamellocytes alone is not enough for the fruit fly larva to kill the wasp egg but also plasmatocytes and crystal cells are needed [16]. The lowered encapsulation response observed here could be therefore due to the misregulation of hemocyte proliferation and/or activation. To examine this, we quantified hemocytes using flow cytometry and discovered that knocking down *edin* in the fat body did not affect lamellocyte numbers but blocked the increase of plasmatocyte numbers after a wasp infection. When hemocytes were activated after an immune stimulus, the banded pattern formed by plasmatocytes was disrupted and the cells were released into the circulation. In addition, it has been previously shown that plasmatocytes develop into lamellocytes after a wasp infection [15, 16]. In our study, sessile plasmatocytes of *edin* RNAi larvae did not leave the sessile bands, and the numbers of circulating plasmatocytes did not change after a wasp infection, yet normal amounts of lamellocytes were formed. Regardless of comparatively normal amounts of lamellocytes, the encapsulation response

was impaired when the sessile plasmatocytes could not be mobilized. Hence, besides giving rise to lamellocytes, plasmatocytes may have yet unknown functions in the encapsulation response that seem to be dependent on *edin* expression in the fat body.

Our results imply that the effect of Edin is non-cell autonomous and that it seems to act as a molecule that signals from the fat body to the hemocytes. Although the humoral and cellular aspects of *Drosophila* immunity are often depicted as separate, several studies have provided evidence of the interaction between hemocytes and the fat body. For example, the antimicrobial peptide response to an *E. coli* infection in *domino* mutants which lack hemocytes, is normal, but these mutants fail to induce *Diptericin* during a gut infection by *Erwinia carotovora* suggesting that hemocytes mediate a signal from the gut to the fat body [26, 27]. In line with these data, Brennan et al. have shown that Psidin acts in the hemocytes to activate the production of Defensin in the fat body [28]. Another example of crosstalk between hemocytes and the fat body is the requirement of *Upd3* expression in hemocytes to activate the JAK-STAT pathway in the fat body of adult flies [29]. Furthermore, in larvae, the production of the cytokine Spätzle by hemocytes is needed for the activation of Toll-mediated AMP production in the fat body [30]. Hemocytes are also mediators of the transport of the nitric oxide from its site of production in the gut epithelia to the fat body, where AMP production via the Imd pathway is activated [31, 32]. However, contradicting data also exist for adult flies showing that the ablation of hemocytes by apoptosis does not affect AMP induction in the fat body [33, 34]. A more recent study has shown that the interaction between the fat body and hemocytes is crucial in controlling tumor cell death [35]. Recently, we also showed that Toll signaling in the fat body controlled hemocyte differentiation and activation, but that it did not play a major role in the immune response against *L. boulardi* as the wasps were able to suppress Toll signaling in the fat body [36]. These examples point to the existence of active tissue-to-tissue signaling that orchestrates appropriate

immune responses against different immune challenges. Based on our results Edin appears to be a key regulator in the cross-talk between the fat body and hemocytes in the context of a wasp infection.

The formation of granulomas in mycobacterial infections in vertebrates has similarities compared to the encapsulation response in *Drosophila*, and we hypothesize that these phenomena may be related. For example, the adult zebrafish responds to a *Mycobacterium marinum* infection by enclosing the infectious foci in granulomas [37, 38]. This reaction also requires the coordinated action of many different cell types. Whether information obtained from genetically tractable model organisms such as *Drosophila melanogaster*, or the zebrafish *Danio rerio*, will lead to a better understanding of the pathophysiology of human tuberculosis remains to be studied.

Materials and Methods

Drosophila stocks

UAS-edin RNAi (CG32185) flies #109528 and #14289 (hereafter called $edin^{109528}$ and $edin^{14289}$) were obtained from the Vienna Drosophila Resource Center. The driver lines used in this study were the fat body-specific driver *Fb-GAL4* [39], the hemocyte-specific driver Hml^{Δ}*;He-GAL4* [40] and *C564-GAL4*, which was obtained from Prof. Bruno Lemaitre (Global Health Institute, EPFL, Switzerland). The *C564-GAL4* driver is expressed in many tissues such as the fat body, lymp gland, salivary glands, gut and brain but not in hemocytes [21].

The hemocyte reporter lines *eaterGFP* (for plasmatocytes) [41] and *MSNF9mo-mCherry* (for lamellocytes, hereafter called *msnCherry*) [42] were obtained from Robert Schulz's laboratory. The lines were crossed to create the *eaterGFP,msnCherry* reporter line. The *eaterGFP,mCherry* reporter was further crossed with *Fb-GAL4* and *edin* $RNAi^{109528}$ to obtain the *eaterGFP,mCherry;Fb-GAL4* and *eaterGFP,mCherry;*$edin^{109528}$ lines. *Canton S* flies were used for RNA extractions.

Wasp infection

Ten *GAL4*-driver virgin females were crossed with five RNAi male flies and allowed to lay eggs at +25°C. w^{1118} flies and *GAL4*-driver virgin females crossed with w^{1118} males and w^{1118} virgin females crossed with RNAi males were used as controls. The flies were transferred daily into fresh vials and the vials containing eggs were transferred to +29°C. On the third day after egg-laying, the larvae were infected with 20 female and 10 male wasps of the *Leptopilina boulardi* strain G486. The larvae were infected for 2 hours at room temperature after which the wasps were removed and the larvae were transferred back to +29°C.

The encapsulation properties were assayed 27-29 hours after the infection and the killing ability of the larval immune system 48-50 hours after the wasp infection. The egg was scored as encapsulated when traces of melanin were found on it. To analyze the killing ability of the *Drosophila* larva, three types of phenotypes were scored. The wasp was scored as killed if a melanized wasp egg or melanized wasp larva without other living wasp larvae was found in the hemolymph, whereas the wasp was scored as living when a living wasp that had escaped a melanized capsule was present or when a living wasp larva without any melanized particles was found in the hemocoel.

RNAi extraction from larvae and fat bodies

Eight to ten *Canton S* larvae per sample were snap frozen on dry ice at 0 hours or 3 hours after the wasp infection. The fat bodies were dissected in 1x PBS 24 hours after the wasp infection and kept on ice. Both larvae and fat bodies were homogenized in TRIsure reagent (Bioline, London, UK) and total RNAs were extracted according to the manufacturer's instructions.

Quantitative real-time PCR

Quantitative RT-PCR was carried out using the iScript One-Step RT-PCR kit with SYBR Green (Bio-Rad, Hercules, CA, USA) and the Bio-Rad CFX96 (Bio-Rad) instrument according to the manufacturer's instructions. Results were analyzed with the Bio-Rad CFX Manager software version 1.6. *Actin5C* was used as a housekeeping gene. The following primers were used: Forward 5'-CTCGTGTCCTGCTGTCTG-3' and reverse 5'-GCCTTCGTAGTTGTTCCG-3' for *edin* and forward 5'-CGAAGAAGTTGCTGCTCTGG-3' and reverse 5'-AGAACGATACCGGTGGTACG-3' for *Actin5C*.

Microscopy

Drosophila larvae were imaged using 3rd instar larvae 27-29 hours after the wasp infection. The larvae were washed three times in H_2O and embedded on microscope slides in a drop of ice-cold glycerol. The larvae were immobilized at -20°C before imaging. The Zeiss ApoTome.2 was used for live imaging of larvae. For hemocyte imaging, the larvae were washed three times in H_2O, and the hemocytes were bled into 1 x PBS 48-50 hours after the wasp infection. Uninfected controls of the same age were also used. The hemocytes were let to adhere to the glass surface of a microscope slide for 30 minutes, after which they were fixed with 3.7 % paraformaldehyde for 5 minutes. The samples were washed with PBS and mounted with the Prolong Gold Anti-Fade reagent with DAPI (Molecular Probes). Hemocyte imaging was carried out with the Zeiss AxioImager.M2 microscope with Zeiss AxioCam and the Zen Blue 2011 software.

Quantification of larval hemocytes with flow cytometry

Infected and uninfected larvae carrying the *eaterGFP,msnCherry* reporter constructs were bled into 1 x PBS with 8 % BSA to obtain the hemocytes. The samples at the 27-29 hour time point were grouped into uninfected and infected larvae. Flow cytometry was used to detect *eaterGFP*-positive and *msnCherry*-positive cells in these samples. The Accuri C6 flow cytometer (BD, Franklin Lakes, NJ, USA) was used to run the samples, and the data was analyzed using the BD Accuri C6 software. The gating strategy is explained in Figure S1.

Statistical analyses

An independent samples two-tailed T-test, with unequal variances assumed, was used to analyze statistical differences between samples. Statistical analyses were carried out using Microsoft Office Professional Plus Excel 2013. The threshold for statistical significance was established as $p<0.05$.

Acknowledgements

We thank A. Kleino, H. Myllymäki, T. Merinen and T. Myllymäki for technical assistance and other members of the MR and DH laboratories for helpful discussions. In addition, we thank the VDRC for the *edin* RNAi lines, R. Schulz for the *eaterGFP* and the *MSNF9mo-mCherry* lines and B. Lemaitre for the *C564-GAL4* line. The fly work was done at University of Tampere Drosophila Core Facility funded by Biocenter Finland and imaging was carried out at the University of Tampere Imaging Core Facility.

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Supporting Information

Figure S1

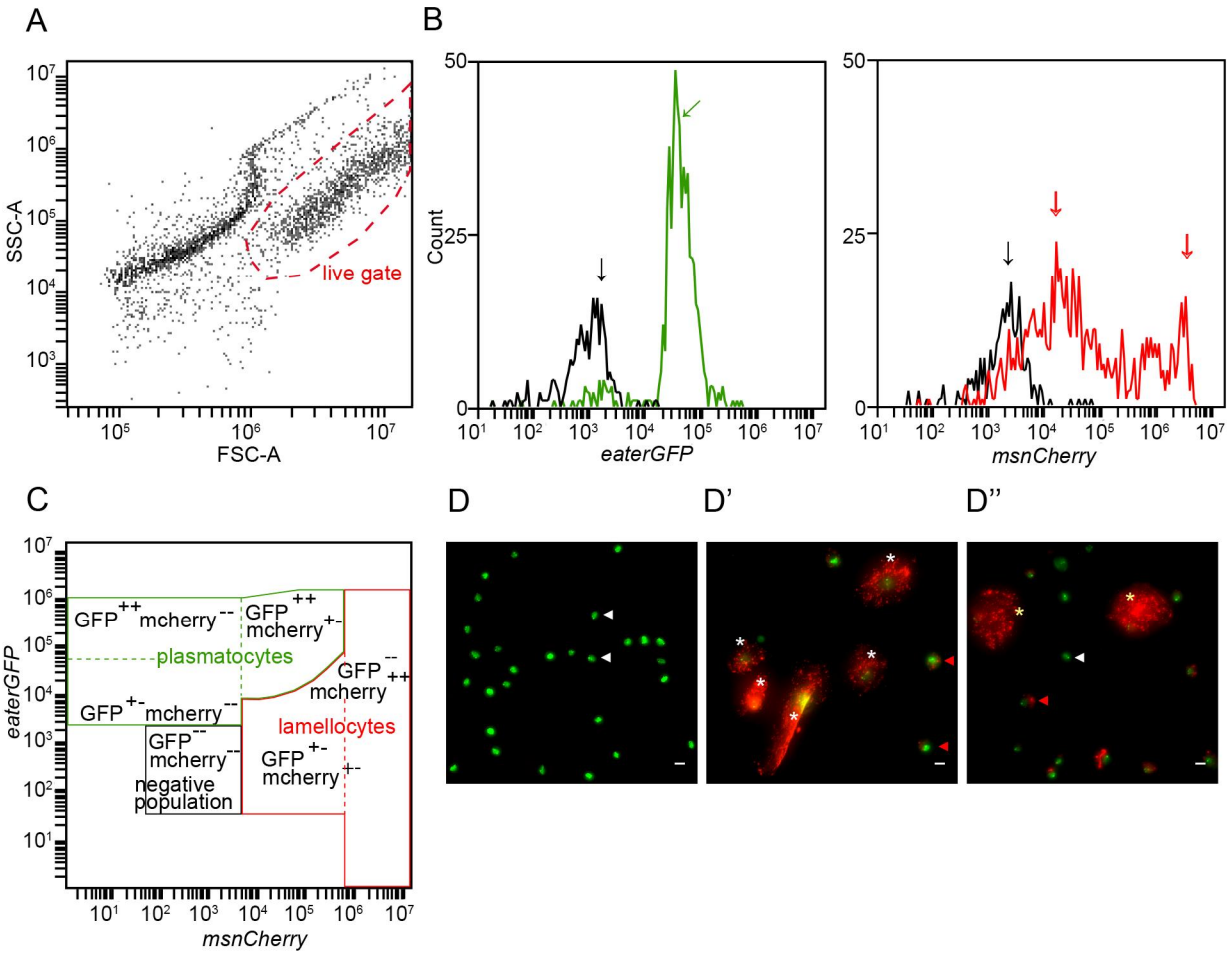

Figure S1. Gating strategy for flow cytometry with the dual reporter *eaterGFP,msnCherry*. (A) Scatterplot of FSC-A against SSC-A on a logarithmical scale. Hemocytes (red dashed ellipsoid) can be readily distinguished from debris. **(B)** Overlay histograms of cells containing neither of the fluorophores (black line and black arrows), *eaterGFP*-only (green line and green arrow), and *msnCherry*-only (red line and red arrows) hemocytes. Fluorescent spillover of the GFP signal into the mCherry detector was corrected by subtracting 8.5 % of the GFP signal. Non-fluorescent hemocytes were detected at low fluorescent intensity that was attributed to autofluorescense. *EaterGFP* had a one maximum peak, whereas *msnCherry* had two peaks. The fluorescent maximum from 10^6 to 10^7 were lamellocytes, the lower intensity peak represented *eaterGFP* and *msnCherry* double positive cell populations. **(C)** Gating strategy with intensities of cell types based on the *eaterGFP* and *msnCherry* expression. The gating strategy was worked out by a timeline experiment (Anderl & Vesala *et al.* unpublished) where the expression pattern of the dual reporter construct in blood cells of infected and age-matched control larvae was followed every second hour during a time course of 50 h after infection with *L. boulardi G486*. We identified five separate cell populations with varying GFP and mCherry expression

and a non-fluorescent negative population. In order to reduce complexity in the current study, we grouped $GFP^{++}mCherry^{--}$, $GFP^{+-}mCherry^{--}$ and $GFP^{++}mCherry^{+-}$ as plasmatocytes and $GFP^{+-}mCherry^{+-}$, and $GFP^{--}mCherry^{++}$ as lamellocytes. All cells grouped as plasmatocytes had plasmatocyte morphology and expressed the plasmatocyte marker *eaterGFP*. Lamellocytes had lamellocyte morphology and expressed *msnCherry*. The dashed lines illustrate the fluorescent intensities of the five distinct blood cell populations. **(D-D'')** Hemocytes grouped as plasmatocytes had plasmatocyte morphology and expressed *eaterGFP* (D, white arrowheads) and *msnCherry* in small granules (D', D'', red arrowheads). Hemocytes grouped as lamellocytes were large and irregularly shaped and expressed *msnCherry* (D'', yellow stars), but also had residual expression of *eaterGFP* (D', white stars). The same representative images are shown in Figure 3A-B'. Scale bars are 10 μm.