Toll-mediated cellular immune response in
*Drosophila melanogaster*

Martin Rudolf Schmid

*Diplom Biologe*
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Martin Rudolf Schmid, *Diplom Biologe*
Cover: Fluorescence microscope image of a Drosophila larva
“Bugs aren't going to inherit the earth.  
They already own it.  
It's time to make peace with the Landlord.”

Dr. Thomas Eisner, Harvard University 1989

Dedicated to my family
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I) SCHMID MR, Anderl I, Valanne S, Vo H, Yang H, Kronhamn J, Rusten TE, Hultmark D: Genetic screen in Drosophila larvae links ird1 function to Toll signaling in the fat body and hemocyte motility. Manuscript


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Important abbreviations used

DNA  deoxyribonucleic acid
RNA  ribonucleic acid
AMP  antimicrobial peptides
GFP  green fluorescent proteins
ORF  open reading frame
UAS  upstream activating sequence
GAL4  galactose
RNAi  RNA interference
dorsal vessel
LG  lymph gland
cortical zone
MZ  medullary zone
PSC  posterior signaling center
proPO  pro-Phenoloxidase
PO  phenoloxidase
wt  wild type
kb  kilo base pair
Rab  ras-like genes in rat brains
GTPase  guanosine triphatase
LAMP1  lysosomal-associated membrane protein 1
PI3P  phosphatidylinositol-3-phosphate
PI3K  phosphoinositide 3-kinase
vps  vacuolar protein sorting
ird1  immune response deficient 1
Summary

Insects are amongst the most abundant and diversified multi-cellular organisms on earth. As pollinators of the vast majority of our food crops their socio-economic value is hard to overestimate. Although many pest and pathogens of the honeybee have been known for decades, we still fail to explain the huge losses of honeybee colonies in recent years.

At the beginning of my PhD studies, I investigated the effect that senescence and the age-related caste dimorphisms have on two basic parameters of the adult honeybee’s immune system, namely blood cell concentration and the activity of the phenoloxidase cascade. Realizing the limitations of working on an organism for which (at the time) no sequenced genome or molecular tools were available, I switched labs to work on Drosophila melanogaster.

The fruit fly has proven to be a particularly useful model system to identify and study genes critical for both the innate immune response itself, as well as the signaling pathways regulating it. For the main part of my thesis, I used the tissue-specific expression of fluorescent markers to visualize segmentally aligned bands of sessile blood cells in the Drosophila larva. This phenotype is disturbed in larvae heterozygote for a gain-of-function mutation in the Toll pathway called Tl10b. In a genetic screen, I scored the ability of genomic mutations to modify the Tl10b loss of bands phenotype. I identified five genomic regions that suppressed the disturbed band pattern of sessile blood cells, and in three of these regions I mapped down this phenotype to single gene level. Two genes are involved in intracellular vesicle trafficking (Rab23 and ird1) and one is activated at the onset of metamorphosis (hdc).

To confirm the experimental model, I tested the role of another negative regulator of the Toll pathway. I used tissue specific GAL4 fly lines to express RNAi silencing constructs targeting Gprk2 expression in vivo. This led to an unexpected and novel discovery. Even though blood cells give rise to the most apparent phenotypes in the Tl10b larva, the main source for the immune signal is the fat body. This indicates that besides the humoral response, also in cell based immunity this organ plays a major role. Based on this finding, I could show that the modification of Tl10b blood cell phenotypes caused by loss of ird1 expression are due the role this gene plays in autophagy cell motility.

The improved understanding of these basic and evolutionary highly conserved mechanisms will undoubtedly help in fending off infectious disease in both man and honeybees in the future.
Preface: why study insects?

Of all organisms that inhabit earth, insects are the most dominating. They can be found in every terrestrial ecosystem, except for very cold environments and high altitudes, and occupy almost every ecological niche imaginable. To achieve this, they had to diversify during evolution into numerous different forms. Natural historians (both amateurs and professionals) have tried to come to terms with this diversity by subdividing insects artificially into orders, families, sub-families, genera and species for centuries. The fact that there are over 350,000 described beetle species alone compared to just 4000 mammalian ones, illustrates that this was a huge effort accompanied by much controversy and revisions. However, this task is far from completed. As researchers penetrate into so far unreachable or unnoticed environments such as the rainforest canopies (Wardhaugh, 2014), they seem to be faced with an ever-increasing number of so far unknown or undescribed animals of the phylum insecta. Besides their apparent beauty and the human urge to make novel discoveries, what drives this continuous interest for these six-legged creatures?

Insects are important vectors for human diseases such as malaria or the African trypanosomiasis, which are responsible for millions of lost lives each year and have devastating effects on the socio-economic growth of many third world countries (World Health Organization, 2004). Besides being a pest to humans, their livestock and crops, they also provide irreplaceable services as pollinators and produce valuable products such as honey and natural wax. It has been noted that out of 100 crop species, 71 benefit from pollination (Food and Agriculture Organization of the United Nations, 2004). Besides increasing crop quality and quantity (Klein et al., 2007; Gallai et al., 2009; Klatt et al., 2014), certain fruits and nuts on which human health and diet depend on would not develop at all without the help of insect pollinators (Seeram, 2008; Eilers et al., 2011). Yet the due to the destruction of natural habitats and the usage of pesticides, many insect species may go extinct before we ever had a chance to notice and study them (Potts et al., 2010). Although economically of little importance in the wild, the fruit fly Drosophila melanogaster has established itself as an invaluable laboratory organism to unravel the basic principles of heredity, development, immunity and neurology.
**Drosophila melanogaster as a model system**

**Historic perspectives**

**Drosophila genetics**

Ever since Dr. Thomas Hunt Morgan published his groundbreaking paper entitled “Sex limited inheritance in *Drosophila*” (Morgan, 1910), the fruit (or vinegar) fly has been the working horse for hundreds of research labs around the world. From the original objective to identify the basis of a naturally occurring mutation causing discoloration of the eye or malformation of the wing, Morgan and his most famous students Calvin Bridges, Alfred Henry Sturtevant and Herman Joseph Muller went on to unravel the principal of chromosome based inheritance (Lewis, 1998). The impact of this discovery on the scientific world and on our understanding of nature in general, are probably matched only by Darwin’s theory of natural selection. After Morgan succeeded through inbreeding to create a fly line consisting of white-eyed individuals only, it was evident that the gene responsible for the trait must be carried on the X chromosome as it occurred in both males and females (Green, 2010). According to the multiple allele hypothesis formulated by Sturtevant in 1913, genes were originally thought as being fixed to their chromosomal position (cited in Lewis, 2004). The first proof that intragenetic recombination exists is often attributed to a report from C.P. Oliver in 1940, where he describes the occurrence of wild type flies produced by females heterozygote for a mutation causing rough eyes (Oliver, 1940). Others accredite this great achievement to the editor of the famous “green book” on the *Biology of Drosophila* (Demerec, 1950), Croatian born geneticist Miroslav Demerec (Lewis, 2004). Sturtevant, after realizing that only some of the discovered sex-linked mutations were interchangeable but others were not, created the first genetic linkage map using the recombination frequency as a measure for genetic distance (Lewis, 1998). After founding his own lab, Muller set about to find artificial ways to cause inheritable mutations. He discovered that by irradiating *Drosophila* sperms with X-rays he could cause mutations in a dose dependent matter. This treatment could also change the linear alignment of genes by bringing about fragmentation, translocations and inversions (Crow, 2005). Besides becoming a Nobel laureate like his mentor Morgan, this has earned Muller a place in the hearts of all Drosophilist’s that depend on balancer chromosomes for their crossing schemes.
Following in their footsteps Christiane Nüsslein-Volhard and Eric Wieschaus used systematic chemical mutagenesis screens to identify genes regulating neurogenesis and embryonic development. Like the adult, *Drosophila* embryos and larvae have easily recognizable features such as segments or bristles at particular positions of their bodies (Fig. 1). When flies were mutagenized, their offspring showed abnormalities that were termed according to their appearance i.e. as Krüppel (*Kr*), Windbeutel (*wbl*) or Spätzle (*spz*) (Nüsslein-Volhard et al., 1987; Nüsslein-Volhard and Wieschaus, 1980; Nüsslein-Volhard et al., 1984). Their revolutionary approach led to the discovery of multiple pathways components that control early development in both fly and play a critical role in wingless, hedgehog and Tumor growth factor signaling (Bellen et al., 2010). Despite the difficulties that many fellow researchers had with the pronunciation of the German names given to the genes identified by Nüsslein-Volhard and Wieschaus, their achievement was awarded with the Nobel Prize in 1995. All this work culminated in the complete sequencing of the first multicellular organism, the fruit fly *Drosophila melanogaster* in the year 2000 (Adams et al., 2000).

**Milestones of *Drosophila* immunity research**

The study on the *Drosophila* immune system goes back to the work of the lab of Hans Boman in Umeå, Sweden. He and his team could show in 1972 that flies vaccinated with *E. cloaca* had an improved survival when subsequently injected with other bacteria compared to their non-primed counterparts (Boman et al., 1972). Later it became clear that this is due to the actions of antimicrobial peptides. The first antimicrobial peptide to be identified was Cecropin named after the *Cecropia* moth where it was discovered (Hultmark et al., 1980). Afterwards antimicrobial peptides were shown to be conserved in vertebrates (Ganz et al., 1985; Selsted et al., 1983) and plants (Cammue et al., 1992). The organ chiefly responsible for the production and secretion of antimicrobial peptides in insects was identified to be the fat body, the insect’s equivalent of the liver in humans (Hultmark et al., 1980). Cecropins were later also found in *Drosophila* (Kylsten et al., 1990; Tryselius et al., 1992) together with Diptericin (Wicker et al., 1990), followed by Drosocin (Bulet et al., 1993), insect Defensin (Dimarcq et al., 1994), Attacin (Åsling et al., 1995), Metchnikowin
(Levashina et al., 1995) and Drosomycin, which is active against fungi (Fehlbaum et al., 1994). The characterization of the genes coding for antimicrobial peptides commenced by cloning the coding DNA of the cecropin (Kylsten et al., 1990) and diptericin loci in Drosophila (Reichhart et al., 1992).

It was Ingrid Faye and her student Sho-Cong Sun who reported in 1991 for the first time, that in the vicinity of genes coding for an antimicrobial peptide in H. cecropia motifs could be found with a similar consensus sequences like those recognized by members of the NF-κB transcription factors family in mammals (Sun et al., 1991). Analogous promoter sequences were later identified to initiate the transcription of i.e. the Cecropin and Diptericin gene in Drosophila (Engström et al., 1993; Sun et al., 1991). Dorsal, a member of the NF-κB protein family had originally been identified due to its role in embryonic development, namely patterning the dorsoventral axis in the early embryo (Steward, 1987; Steward and Nusslein-Volhard, 1986). During the course of a genetic screen that was devised by Nüsslein-Vollhard, eleven more genes were discovered to be important for this process. Beside the nerve growth factor related cytokine Spätzle (Chasan and Anderson, 1989; Weber et al., 2003) and the death domain containing proteins Tube and Pelle (Towb et al., 2001), the screen identified the transmembran receptor Toll (reviewed in Belvin and Anderson, 1996), named after the German word for great Drosophila (Tl) proved to have sequences homologies to the interleukin-1 receptor, involved in the acute phase response and inflammatory immune responses in mammals (Gay and Keith, 1991; Hashimoto et al., 1988; Ferrao et al., 2012).

However, in contrast to the expectations dl was shown to be dispensable for the induction of Diptericin expression in response to a lipopolysaccharides challenge (Reichhart et al., 1993). Later another member of NF-κB was identified, that like dl translocates to the nucleus of fat body cells upon infection but is not expressed at the embryonic stage (Ip et al., 1993). Named Dorsal-related immunity factor (Dif), it was found in a low stringency hybridization screen of a Drosophila DNA library that used conserved regions of the dorsal (dl) domain as bait (Ip et al., 1993). In the same study it was shown that Dif binds to the κB motif in a Cecropin promotor (CecA1) by gel shift assays. It was also noticed that in larvae carrying the Tl<sup>rob</sup> allele coding for a constitutively activated form of the receptor (Erdelyi and Szabad, 1989) both Dif and dl were nuclear even in absence of a microbial infection (Ip et al., 1993). This indicated for the first time that a connection might exist between the Tl receptor and the actions of NF-κB-like proteins in immunity. The finding that tissue culture cells
over-expressing $IT^{10b}$ showed an increased CecA1 expression (Rosetto et al., 1995) further validated this notion.

In the following year it was shown by the lab of Jules A. Hoffmann, that *Drosophila* adults carrying *IT^{10b}* or being deficient for the *dl* inhibitory protein Cactus (Kidd, 1992; Geisler et al., 1992) showed constitutive Drsomycin expression even in absence of an immune challenge (Lemaitre et al., 1996). Coherently, the same study showed that *spz, Tl, tub* and *pll* deficient mutant adults were compromised in their ability to respond to a bacterial infection by Drsomycin expression. Lastly, flies carrying loss-of-function alleles for *Tl* or the recessive mutation immune deficiency (*imd*, (Lemaitre et al., 1995)) rapidly succumbed to bacterial or fungal infections (Lemaitre et al., 1996). Later studies showed that for Drsomycin expression after an immune challenge the actions of *Dif* rather than *dl* are essential (Manfruelli et al., 1999; Meng et al., 1999; Rutschmann et al., 2000) and that the Drsomycin gene is also independently induced by another NFκB like transcription factor, Relish (Hedengren et al., 1999). For his contributions in establishing the importance of the *Tl* signaling pathway for the activation of the innate immune system Jules A. Hoffmann shared the Nobel Prize in physiology or medicine in 2010 with Bruce A. Beutler and Ralph M. Steinman.

### Why *Drosophila*?

The fruit fly has many properties making it the ideal lab organism. Compared to other animal model systems *Drosophila* is much less costly and time consuming. This is due to the flies frugal life style, short reproduction cycle and fast development. Also in comparison with mice or rats, the risk of injury through bites or scratches is much reduced for the researcher. While the need for ethical approvals and meeting minimal requirements for keeping and testing vertebrates such as zebra fish and mice has increased dramatically in the Western World over the past decades, *Drosophila* has remained in the blind spot of this development. Possibly this due to the fact that a fly is not considered an animal at all according to most national law systems, including the Swedish. This attitude may be attributed to the widespread vertebrate chauvinism amongst people.

The anatomical features of the adult fly such as wings, bristles or form and color of the eye are not only esthetically appealing, but also easily recognizable even at low magnifications. The formations of the body pattern in the *Drosophila* embryo are so precise that it hardly differs from individual to individual (Jaeger, 2004; Liu et
Less prevalent at later developmental stages, it is this reproducibility that made the *Drosophila* embryo the most important system to analyze transcriptional regulation of metazoan genes (Gregor et al., 2014). Also, manipulations such as removal or addition of single genes, their products or mutagenesis by chemicals or X-ray can often affect these physical characteristics in a direct manner. As recombination does not occur in male *Drosophila* flies and by using balancer chromosomes, it is relatively easy to establish true breeding stocks for any newly discovered mutations.

Balancer chromosomes are unique to the *Drosophila* model system. Besides carrying many genetic alterations such as inversions or translocations that inhibit recombination during meiosis even in females, they also harbor recognizable dominant marker mutations affecting i.e. the form or length of wings and bristles. By scoring flies against these markers it is possible to follow even recessive mutations, which are usually invisible in heterozygote conditions (Greenspan, 2004). Newly established mutant strains can then be used to investigate null alleles, hypomorphs, neomorphs and antimorphs to rapidly assess the function of the mutated gene (Bellen et al., 2010).

The ease and speed of assays conducted with *Drosophila* makes it possible for researches to conduct large-scale screens for hundreds of genes in just a few weeks. By this they can unravel whole signaling pathways helping to answer fundamental questions of biology, such as what genes and processes guide development, immunity or behavior. In contrast to using cell culture, this information is not limited to single cells but can help to explain complex processes such as development of organs and their communication with each other. Also despite its relative small size, the fly possesses a complex nervous system capable of learning and memory formation (Bilen and Bonini, 2005). Although anatomically quite different from the human brain, many important features for the development and function of neuronal systems can be found in the fly’s brain (Whitworth et al., 2006).

It has been estimated that around 75-80% of the genes connected to human diseases have orthologs in *Drosophila* (Cauchi and van den Heuvel, 2006; Reiter et al., 2001; O’Kane, 2003). One example illustrating this point is the Notch signaling pathway. Notch was originally discovered as a mutation causing wing malformation (Morgan, 1910). Later it was found that the human NOTCH and other components of this pathway that are conserved in both fly and man are involved in leukemia (Kopan and Ilagan, 2009). Also the Hedgehog (*hg*), Wingless (*wg*), Runt and Hippo pathways were first discovered in *Drosophila* and have now been implemented in
cancer formation in humans (Geissler and Zach, 2012; Harvey et al., 2013). In contrast to humans and other mammals *Drosophila* has a rather small genome located on just four pairs of chromosomes. This may be the reason for the reduced genetic redundancy of the components of central signaling pathways, making forward genetic screens in *Drosophila* so successful. With the growing number of labs and researchers using *Drosophila* as a model, more and more tools become available that can be used to answer research questions that may be posed by future generations of scientists.

**Genetic tools and techniques**

Apart from the classical approaches of altering *Drosophila* genome by X-ray or chemical mutagenesis, in the last decades methods have been developed that are more specific and less hazardous for the experimenter. Barbara McClintock originally identified mobile DNA elements (or transposons) in maize in the early 1950’s (Green, 2010). After 15 years their existence could be confirmed in the fruit fly as the first organism other than corn (Green, 1967). Since than at least a dozen of specially engineered transposons have been integrated into the *Drosophila* genome, i.e. to cause disruption of gene function at their insertion side (reviewed in Bellen et al., 2004). For this insertional mutagenesis approach transposable elements were artificially created carrying easily scorable genetic markers, such as drug resistance or dominant mutations. This made it possible to identify flies carrying the transposons by their altered morphological traits (such as eye color) throughout complex crossing schemes using balancer chromosomes.

Besides scorable markers, specialized transposons can carry sequences coding for *LacZ* (Hartenstein, 1992) or green fluorescent marker proteins (GFP) from the jellyfish *Aequorea victoria* (Morin et al., 2001; Berghammer et al., 1999; Morin et al., 2001). When inserted into the proximity of endogenous enhancers these “trap” constructs called *P*-elements visualize the expression of adjacent genes and/or can cause their over expression (O’Kane and Gehring, 1987; Bier et al., 1989; Rørth, 1996). The sequence coding for the enzyme transposase that caused the original insertion of the *P*-element can be re-introduced through a cross inducing the re-mobilizations of the transposable element. This can lead either to a precise excision event restoring wild-type conditions, or an imprecise excision in which the *P*-element takes adjacent DNA sequences with it creating new small deletions (Preston et al., 1996; Gray, 2000).
Another more precise method to cause DNA double strand breaks by P-element mobilization became available when the FLP/FRT system was integrated into Drosophila. Flip-recombinase target side (FRT) containing P- and piggyBac elements have to be inserted separately at different sides of the same gene on two homologous chromosomes (Thibault et al., 2004). When the enzyme flippase (FLP) is expressed in these flies the DNA sequence between the two FRT sides is exited in trans to create deletions covering only the targeted gene (Golic et al., 1997). The same basic approach has been used to create hundreds of deletions together covering the majority of the Drosophila genome. These fly stocks were created in the same genetic background and each carry single deletions with known breakpoints. The strains have been made publicly available by the functional genomics company Exelixis and the DrosDol consortium (Parks et al., 2004; Ryder et al., 2004). Besides making it possible to rapidly carry out genome wide deletions screens to identify genes affecting the phenotype of choice, these projects also provided the methods and tools to create new deletions with desired breakpoints. The FRT/FLP system can also be used to create Drosophila mosaics (Theodosiou and Xu, 1998). These animals contain cells that are composed of cells of different genotypes that can be distinguished by the expression of specific fluorescent reporter constructs (Lee and Luo, 2001). This makes it possible to visualize the effects of experimental manipulations in mutant and wild-type cell lying next to each other in the same organ.

A more recent way to create targeted deletions and insertions was developed by Gong and Golic (2003). Termed homologous recombination this technique aims at introducing a modified sequence next to or into a chromosomal target gene using an engineered copy of an exogenous piece of DNA molecule called the donor (Bi and Rong, 2003). The donor carrying a DNA homologous to the target sequence but with a point mutation or a reporter gene is inserted randomly into the genome via a P-element that also carries an I-SceI recognition side. Through the actions of FLP and the rare cutting endonuclease I-SceI the P-element is released forming a closed circle and a double strand break is induced. The linear DNA piece moves freely to its target sequence where it undergoes homologous recombination integrating itself next to or into the open reading frame (ORF) of the endogenous gene (ends-in) (Bi and Rong, 2003; Rong et al., 2002). The result is a tandem partial duplication of the target gene in which both copies are non-functional as they each lack a portion of the gene (Gong and Golic, 2003).

In the past couple of years two new genetic manipulation techniques have emerged that are applicable to almost all animals and plants. The TALEN and
CRISPR systems named after the acronyms transcription activator-like effector nuclease and (Yu et al., 2014) clusters of regularly interspaced short palindromic repeats, respectively have both been adapted to Drosophila to generate genetic insertions/and or deletions (Bassett et al.; Liu et al.; Yu et al.). TALENs are customly engineered for every target locus and contain repeats of DNA-binding domains and a Fok I nuclease domain. However, as the Fok I nuclease only cuts genomic DNA when present as a dimer (Bitinaite et al., 1998) two TALENs have to bind nearby in a head-to-head manner (Katsuyama et al., 2013). The created double strand break again stimulates the endogenous DNA repair machinery inducing (non)-homologous recombination with exogenously provided DNA sequences causing their integration into the genome. In contrast TALEN-mediated genetic modifications where the target binding specificity is defined by DNA-protein contact, the CRISPR system relies on a single guide RNA (Miller et al., 2011). The guide RNA binds directly to the DNA of the target gene identifying it for being cut by the Cas9 nuclease (Gratz et al., 2013). Like with TALEN the double strand break when imperfectly repaired creates mutations or introduces foreign sequences into the target gene.

881 amino acid long protein (galactose), which binds to specific sites called Upstream Activating Sequences (UAS) that control the expression of adjacent genes (Duffy, 2002). The GAL4 sequence has been cloned into various P-elements, that when
integrated into the *Drosophila* genome close to an enhancer element induces the production of the GAL4 protein in the same temporal and spatial fashion as an endogenous gene (Duffy, 2002). Thousands of these stocks called drivers have been created by the Drosophila community (Bellen et al., 2004; Hrdlicka et al., 2002) (Rørth, 1996; Lukacsovich et al., 2001; Horn et al., 2003), making it possible to induce GAL4 production at almost all possible time points of *Drosophila* development and in a huge variety of tissues (Duffy, 2002). GAL4 expression can be easily monitored by crossing driver stocks to responder or reporter lines carrying a UAS element coupled to sequences coding for GFP of lacZ (Duffy, 2002). Alternatively, the UAS element can control expression of sequences coding for toxic or lethal products or genes that trigger programmed cell death (Zhou et al., 1997).

The UAS/GAL4 system can also be used to silence expression of target genes with known sequences by RNA interference (RNAi, Fig. 3). For this an inverted repeat sequence coding for a target gene can be cloned into a modified pUAST vector and inserted into the *Drosophila* genome (Kennerdell and Carthew, 2000). When this fly stock is crossed to a time and tissue specific driver line the transcription of the palindromic sequence causes the formation of an RNA hairpin loop (Carthew, 2006). After cleavage the resulting short interfering-RNAs (siRNA) excite the nucleus as fragments, which are further processed before being incorporated into the ribonucleoprotein complex (RISC). The natural function of this
complex is to survey the cell for foreign nucleic acid usually present at a high copy number and introduced i.e. by viruses (Carthew, 2001; Carthew, 2006). The target mRNA whether foreign or endogenous is recognized by base pairing with the sequence carried within the RISC complex inhibiting protein translation effectively silencing gene expression. Fly stocks carrying single UAS-driven inverted repeats have been created with sequence homologies to almost every protein-coding gene in the Drosophila genome (Dietzl et al., 2007). Most of them have become publicly available (http://stockcenter.vdrc.at) and recently a newer generation of RNAi bearing stock collection was added carrying the insert at a single specific location of the genome (Green et al., 2014).

Life cycle and development of Drosophila

The embryo
At 25°C the life cycle of Drosophila melanogaster takes about 9-11 days from egg-laying till the emergence of the imago (Bodenstein, 1950, and Ashburner et al., 2005), Fig. 4). The fly lays numerous eggs into ripe or damaged fruit on which its larvae will feed after hatching. Before that, in the first three hours after fertilization, the expression of gene products in the Drosophila embryo is governed by cues that have been maternally supplied (Gregor et al., 2014). The nuclear concentrations of these transcription factors differ in their concentration along the anterior posterior axis. This leads to the formations of a body pattern that ultimately changes the fly from the single cell system of the embryo to the highly complex structure of the adult. Earlier in a phase called syncytium the nuclei of the embryo go through 13 rapid, synchronized cell divisions. The resulting 6000 cells migrate to the egg surface but, as they lack plasma membranes, gene products can still diffuse freely throughout the body of the embryo. This is crucial for the establishment of maternal gradients
formed by transcription factors such as bicoid (Crauk and Dostatni, 2005). They activate zygotic patterning genes in specific spatial domains, which together with gap and pair rule genes lay down a bauplan defining the position for future structures of the adult organism. Eventually the embryonic cells complete their cytokinesis forming the cellular blastoderm.

In the next step gastrulation sets in with the ventral layer of cells invaginating resulting in the formation of three distinct germ layers (Lawrence, 1992). In the inner layer called the mesoderm the cells acquire the fate to form the internal organs including muscles, blood, fat body and dorsal vessel (DV). The latter two are the *Drosophila* equivalents of the human liver and heart. The outer layer of the ectoderm will give rise to central and peripheral nervous system, epidermis and trachea. The third layer of cells the endoderm originates from the ingrowth of the anterior and posterior midgut primordia, that when fused in the middle of the embryo will form the midgut (Nüsslein-Völlhard, 2006). The primordia are tailed by one tube each consisting ectoderm that will later form the foregut and hindgut (Lawrence, 1992). The ectoderm and mesoderm stretch along the dorsal and ventral inner side of the embryo, respectively. Simultaneously, the mesoderm rolls into a furrow along the ventral midline forming the germ band (Lawrence, 1992; Nüsslein-Völlhard, 2006). This band of cells will give rise to the main trunk of the embryo that will be easily recognizable due to its segmentation. Before the germ band forms completely neuroblasts plunge in one by one forming an interjacent layer that is the basis for the central nervous system. After completion of the gastrulation process major cell divisions set in and the cells start to differentiate according to their positions in the future organs.

Although prior to an during gastrulation the segmentation of the embryo can be made visible through the expression of certain genes, it is not until the invagination of the germ band that clear morphological subdivisions become apparent. After 10 h the cells along the epidermis start to show evenly spaced grooves demarking the parasegments that to a degree correspond to the mouthparts (G1-G3), the thorax (T1-T3) and the abdominal segments (A1-A8) of the adult fly (Lawrence, 1992). Following the formation of the cuticle the larva hatches 24 h after egg deposition and immediately commences feeding.

**Larval stages**

The *Drosophila* larva is subdivided into 12 segments, consisting of the head with the mouth hooks, three thoracic and eight abdominal segments (Bodenstein,
Each segment is fitted on its outside with multiple rings of chitinous hooks that assist the locomotion of the larva. Despite being composed out of three different layers (outer cuticle, inner cellular epidermis and basement membrane), the larval body is soft and flexible. In addition, it is semi-transparent making it possible to observe internal organs such as fat body and intestine without dissection using a light microscope with moderate magnifications. Besides producing antimicrobial peptides, the fat body acts as the major store of proteins providing energy when the fly is not feeding. The larval development goes through three stages called instars. The first and second instars last 24 h each whereas the final third takes 48 h. Between each stage the larva sheds its skin to account for the enormous increase in body mass acquired through constant feeding.

The larva contains two distinct types of cell lineages. The first one exclusively forms larval structures and becomes increasingly polyploid though chromosome duplications without subsequent cell divisions. This is why, although the larvae increases considerably in size through its three instar stages, the cell number and its basic bauplan remains basically unchanged (Weaver and White, 1995). In contrast the cells making up the imaginal discs remain diploid. These sacs of cells represent the anlage from which the adult structures will be formed. Hormones control their growth throughout larval life and the structural changes they undergo will eventually lead to the histolysis of the larval organs and the genesis of the adult body (Weaver and White, 1995).

The larval heart can be found on the dorsal side. It has the basic shape of a bottle and consists of a broader body at the posterior end (the actual heart) and a long neck stretching till the larval head called the dorsal vessel. In contrast to vertebrates, Drosophila has no veins. Instead the transparent blood called hemolymph enters through several openings of the heart, from where it is pumped through the dorsal vessel to circulate freely between the internal organs. Also the blood of Drosophila transports only nutrients, hormones and the cells participating in the immune response called hemocytes, but no oxygen. Instead air is sucked into an intricately branched network of hollow tubes called trachea by openings in front and in the back of the larva (Nüsslein-Vollhard, 2006). The CNS of the larvae consists of a brain and a ventral chord in rope-ladder-shape. Neuroblasts can be found distributed in a characteristic pattern along the ectoderm innervating the body with stings of nerves and knots. After the cessation of feeding at the wandering stage, the larva emerges from the food in search for a suitable place for its transformation into an adult fly.
**Pupa**

The period from the beginning of the metamorphosis to the eclosion of the adult fly is usually between 4-5 days (Ashburner et al., 2005). During this time the structure of the larva is almost completely dissolved and replaced by the body parts of the adult.

**Adult**

Female flies do not mate readily during the first 12-14 hours after emerging from the pupa. In this time their exoskeleton hardens and the wings unfold and dry. After an elaborate courtship that involves chasing, tapping singing (vibrating wings) and licking (Yamamoto and Koganezawa, 2013), the male injects his sperm into the female where it is store in the spermatheca. Ovulation begins 1.5 h after mating and the mature eggs are inseminated before being deposited on the food where a new cycle of *Drosophila* development begins (Ashburner et al., 2005).

**Immunity of *Drosophila***

Insects are frequently injured, i.e. by parasitic wasps and nematodes (Ffrench-Constant et al., 2007; Schmidt et al., 2001). This is particularly dangerous for the *Drosophila* larva that feeds on fermenting fruits containing numerous microorganisms. It is therefore not surprising that an elaborate array of defence mechanisms has evolved in *Drosophila* protecting both larvae and adult flies from intruders from their hazardous environments. The exoskeleton, trachea and the gastrointestinal tract represent the first barriers protecting the fly from infectious agents. Apart from their physical properties, these structures are known to produce antimicrobial peptides and free radicals to make them inhospitable for microbes attempting colonization (Tzou et al., 2000; Lemaitre and Hoffmann, 2007).

Once these barriers have been breached (for instance through a wound), the invaders are first met by a potent local response geared towards containing the infection. Apart from blood clotting, it is mainly the deposition of melanin at the wound side that hinders the infection from spreading (Theopold et al., 2014). If this fails, invading microorganisms are met by immune responses that can be broadly distinguished into three classes based on their main mediators. The cellular immunity clears the hemolymph from foreign objects by phagocytosis or encapsulation. Bigger object such as parasitoid eggs are engulfed in several layers of
blood cells forming a capsule (Williams, 2007). The humoral immunity relies on antimicrobial peptides that are mainly produced by the fat body but also elsewhere and can directly kill bacteria and fungi. The phenoloxidase based immunity disposes of invaders either directly through production of toxic compounds such as hydrogen peroxide \((\text{H}_2\text{O}_2)\) or indirectly by participating in their immobilization through blood clotting. The melanin produced by PO may also play a role for the encapsulating parasitic wasp eggs (Kounatidis and Ligoxygakis, 2012, and reviewed in Lemaitre and Hoffmann, 2007).

**Cellular immunity**

**Drosophila blood cells**

Compared to mammals with their numerous veins and capillaries, the *Drosophila* circulatory system is rather simple. Also the capabilities of the cells transported within it, the hemocytes, are comparably limited. In stark contrast to mammalian B- and T-lymphocytes fruit fly blood cells are not capable of memory formation by DNA rearrangement and somatic hypermutation (Wang et al., 2014). In contrast to the seven different cell types that can be found in human blood, the fly only has three (Fig. 5) (Shrestha and Gateff, 1982; King et al., 1984).

![Plasmatocytes, Crystal cells, Larvalocytes](image)

*Figure 5. Drosophila blood cell types.*

*Adapted from: Lanot et al. (2001), Dev. Biol., 230:243-257*

Plasmatocytes, a class of small round professional phagocytes, are the most abundant cell type and can be found at every developmental stage (Elrod-Erickson et al., 2000; Rizki, 1978). They remove debris and doomed cells that accrued during the different stages of development (Tepass et al., 1994; Lanot et al., 2001). In the embryo this function of depends on scavenger receptors like Draper, Nimrod C4 and Croquemort (Franc et al., 1996; Manaka et al., 2004; Kurant et al., 2008). In contrast
in larval stages, plasmatocytes are chiefly responsible for ingesting invasive pathogens like bacteria and fungi (Rämet et al., 2001; Kocks et al., 2005). For this, they rely on the recognition of the invaders through cell surface receptors like Eater and NimC1 (Kocks et al., 2005; Kurucz et al., 2007). Plasmatocytes also support the immune response by producing and secreting antimicrobial peptides (Dimarcq et al., 1997), and can possibly directly recognize parasitic wasp eggs (Russo et al., 1996). In addition, they are believed to be a source for signaling molecules that can activate other tissues turning a local to a systemic immune response (Agaisse et al., 2003; Irving et al., 2005a).

Crystal cells, which constitute only about 5% of the hemocyte population in embryos and larvae, are round and similar in size to plasmatocytes (Shrestha and Gateff, 1982; Lebestky et al., 2003). In contrast, however, they contain distinct crystalline inclusions of phenoloxidase processing the melanization reaction necessary for wound closure and encapsulation response in the fly and other invertebrates (Binggeli et al., 2014; Cerenius et al., 2008; Russo et al., 1996).

Lamellocytes are large flat cells of irregular shape, which mediate an encapsulation and neutralization response of objects too large to be taken up by plasmatocytes. Lamellocytes are not found in embryos or adults and usually only appear in larvae after an immune challenge like a parasitic wasp attack, a wounding or prior to pupariation (Rizki and Rizki, 1992; Lanot et al., 2001; Sorrentino et al., 2002; Krzemien et al., 2007; Markus et al., 2005). In collaboration with plasmatocytes they surround the wasp egg and participate in the melanization of the capsule, which is needed for the successful killing of the invader (Williams, 2007).

Haematopoiesis

Like in vertebrates haematopoiesis in Drosophila occurs in two spatiotemporal waves (Holz et al., 2003). The first wave starts in the cephalic mesoderm in the early embryo, generating pro-hemocytes marked by expressing the early GATA transcription factor Serpent (Tepass et al., 1994; Rehorn et al., 1996; Lebestky et al., 2003). These cells start differentiation by expressing the Drosophila friend of GATA, *u*-shaped (Wang et al., 2014; Williams, 2007). Co-expression of *serpent* and *u*-shaped turns on the production of transcription factor Glial cell missing (Gcm) and its isoform Gcm2, committing these cells to the plasmatocyte lineage (Fossett et al., 2001). In contrast, in a sub-population expression of the transcription factor Lozenge antagonizes or suppresses *U*-shaped and Gcm/Gcm2 turning these cells into crystal cells (Bataillé et al., 2005). While about 700
Plasmatocytes migrate along defined routes thereby populating the entire embryo (Wood et al., 2006), the 30 crystal cells remain sessile at the midgut and proventriculus (Lebestky et al., 2000). Together these blood cells form the pool from which the up to 7000 blood cells that can be found in circulation by the end of the third larval instar derive (Fig. 6). Most of these cells retain their proliferating capability (Asha et al., 2003).

The main role of circulating blood cells is however to survey the open circulatory system of the fly (Babcock et al., 2008). As they are constantly pumped through the heart they can by chance encounter damaged tissue or any immunological threat like a pathogen or a wasp egg and immediately participate in a defense reaction. In addition, they can send signals to recruit other hemocytes and activate a general immune response by the fat body. Lamellocytes have been recently shown to derive directly from plasmatocyte by upregulation of serpent and charlatan and down regulation of u-shaped (Honti et al., 2010; Stofanko, 2010). However, their formation is depending on the activation of the larval immune system (Sorrentino et al., 2002; Lanot et al., 2001).

Besides circulating cells, a second population of hemocytes from embryonic origin aligns itself along the integument of the larva and is referred to as sessile cells (Lanot et al., 2001; Holz et al., 2003; Makhijani et al., 2011). They are mostly phagocytic plasmatocytes and some crystal cells (Lanot et al., 2001; Honti et al., 2014) and are distributed in epidermal-muscular pockets already at the first larval stage (Makhijani et al., 2011). By the third instar the proliferating sessile cells have finished to colonize these patches starting from the lateral to both dorsal and ventral sides. These rings of hemocytes form a regular striped pattern on the dorsal side of the larvae (Fig. 6) (Zettervall et al., 2004). Plasmatocytes are a known source of extracellular matrix proteins, which may aid to the attachment of these cells to their compartments (Wood and Jacinto, 2007; Brock et al., 2008; Evans et al., 2003).
However the fact that gentle brush strokes can induce the loss of this pattern proves that these resident plasmatocytes are not immobile but can move laterally between the integument and the muscle layer (Makhijani et al., 2011) and possibly also into circulation. The peripheral nervous system plays a pivotal role in establishing a trophic microenvironment that attracts the mobilized cells back to their resident locations within 30 min after the disturbance (Makhijani et al., 2011). In contrast, when important signaling pathways like JAK/STAT or Toll are artificially activated i.e. by expressing constitutively active forms of receptors using a hemocyte specific Gal4 driver the majority of blood cells are in circulation (Zettervall et al., 2004; Williams et al., 2006; Márkus et al. 2009). This loss of the sessile hemocyte pattern phenotype can also be observed in larvae that have been parasitized by the wasp *Leptopilina boulardi* indicating a biological relevance for this phenotype (Zettervall et al., 2004; Márkus et al., 2009). As the epithelial and muscle layer is penetrated first by the wasps ovipositor, the presence of the sessile cells in this layer may be crucial for the early recognition of the immune challenge.

The second hematopoietic wave takes place in specialized organ called the lymph gland. This blood cell reservoir forms during embryogenesis from the mesoderm in response to Notch signaling (Crozatier and Meister, 2007; Holz et al., 2003). Starting as a single pair of lobes in the embryo and early larval stages, the lymph gland develops by the proliferation of blood cells into a grape shaped structure located anteriorly alongside the dorsal vessel in the third larval instar (Figs. 6 and 7) (Evans et al., 2003; Lanot et al., 2001). Morphologically the first lobes to develop during embryogenesis can still be recognized in later larval stages for being the foremost and biggest structures of the organ.

Figure 7. Third instar larval lymph gland expressing Hml-Gal4,UAS-GFP. Pericardial cells (PC), dorsal vessel (DV), posterior signaling center (PSC), medullary zone (MZ), cortical zone (CZ).
Consequently these lobes are referred to as primary and the smaller ones (interspersed by nephrocytic pericardial cells) as secondary lobes.

In healthy larvae the lymph gland is dominated by blood cell progenitors that start to differentiate with the beginning of the third instar larvae (Lebestky et al., 2000; Jung et al., 2005). This leads to the formation of mature plasmatocytes, crystal cells and few lamellocytes in the primary lobes. Based on the localization of these differentiated cells, expression of certain markers and morphological features, the primary lymph gland lobes are typically subdivided into three major compartments: the cortical zone, the medullary zone and the posterior signaling center (Fig. 7) (Jung et al., 2005; Crozatier et al., 2007; Krzemień et al., 2007). The stem cell-like character of the densely packed progenitor cells contained within the medulla is maintained by the activity of the JAK/STAT, hedgehog and wingless pathway (Krzemien et al., 2010; Mandal et al., 2004; Tokusumi et al. 2003; Sinenko, 2009). The expression of the transcription factor Collier in cells localized in the posterior signaling center is controlled by the Serrate/Notch signaling pathway. The cells of the posterior signaling center selectively express Hedgehog and Serrate transcription factors (Lebestky et al., 2003; Krzemien et al., 2007; Mandal et al., 2007) and are believed to exert its control over the undifferentiated cells of the medullary zone through connecting filopodia (Tokusumi et al., 2010). Like for the differentiation of the circulating blood cell population, transcriptional inputs by Serpent, U-shaped as well as Suppressor of Hairless exert a role in the lymph gland, where they are in turn controlled by the hedgehog-signaling pathway (Tokusumi et al., 2010).

Premature differentiation of the hemocytes contained within the lymph gland can be induced naturally by wasp infection or artificially through perturbation of the above-mentioned signaling pathways or by increasing reactive oxygen species levels (Owusu-Ansah and Banerjee, 2009; Tokusumi et al., 2009; Crozatier and Meister, 2007). However in contrast to previous claims (Lanot et al., 2001; Krzemień et al., 2007), hemocytes originating from the lymph gland do not seem to play a significant role in the larva itself. Their release from the lymph gland into circulation comes possibly too late to be important for the encapsulation of the wasp egg (Márkus et al., 2009) and therefore their sole role may be involved in the deposition of the larval structures during metamorphosis (Lanot et al., 2001). The main burden to fend off immunological challenges to the larva may therefore rest on the circulating and sessile population of hemocytes, aided by antimicrobial proteins and peptides produced mainly by the fat body. No haematopoiesis has so far been reported to occur in the pupa or the adult fly (LeMAître and Hoffmann, 2007). The basic
organization and the signalling pathways involved in maintaining blood cell identity within the lymph gland bear similarity to hematopoietic stem cells niches in vertebrates making it a suitable model (Orkin and Zon, 2008).

**Humoral immunity**

This arm of the *Drosophila* defense system is activated when the immune reaction has become systemic, involving all immune relevant tissues. Antimicrobial peptides are powerful effector molecules that (when secreted by the fat body into the hemolymph) are capable to directly destroy microbial cell walls (Bulet et al., 1999). However, compared to the immediate phagocytosis reaction of plasmatocytes the production of antimicrobial peptides is transcriptionally regulated and therefore delayed (Rämet et al., 2002; Haine et al., 2008). The antimicrobial peptides identified in *Drosophila* include Metchnikowin, Defensin, Cecropin, Diptericin, Attacin and Drosomycin (Imler and Bulet, 2005). In *Drosophila*, Toll gives name to one of at least four signaling pathways known to be involved in immune responses, the other ones being the immune deficiency (IMD) (Corbo and Levine, 1996; Lemaitre et al., 1995), JNK and JAK/STAT pathway (reviewed in Ganesan et al., 2011). Only the first two will be described briefly in the following, as they are most relevant for the present thesis.

**Immune signaling pathways**

**Toll (Tl)**

In contrast to some mammalian Toll-like receptors that directly recognize bacterial lipopolysaccharides or lipopeptides (Taguchi et al., 1996; Poltorak et al., 1998), in *Drosophila* invading microbes are detected with the help of at least 12 different peptidoglycan recognition proteins (PGRP, Fig. 8). Depending on if they are cell bound or secreted, they are broadly...
subdivided into the groups PGRP-S or PGRP-SL, respectively (Werner et al., 2000; Jung et al., 2001; Michel et al., 2001). Together with the misnamed gram-negative binding proteins (GNBP) (Kim et al., 2000), these proteins form complexes that activate an extracellular proteolytic cascade involving the modular serine protease (ModSP) and serine protease Grass (Buchon et al., 2009; Kambris, 2006). Independently the serine protease Persephone (Psh) can cause the activation of Toll signaling after sensing virulence factors such bacterial or fungal proteases (El Chamy et al., 2008; Gottar, 2006). The serine protease inhibitor Necrotic can block the activity of Persephone adding another level of control (Robertson et al., 2003). In addition, protease Spirit, Spheroide and Sphinx1/2 are needed for the activation of the immune response in case fungal or bacterial microorganisms invade the host (Kambris et al., 2006). All these processes culminate in the cleavage of the neurotrophin-like cytokine Spätzle (Weber et al., 2003; Mizuguchi et al., 1998) from its pro-domain by the Spätzle Processing Enzyme (SPE) (Ligoxygakis et al., 2002; Gobert et al., 2003; Jang et al., 2006).

The binding of two Spätzle dimers by their N-terminus to a single Tl leads to the transmission of the immune signal by a conformational change of transmembrane receptor (Fig. 9) (Gangloff et al., 2008). This activates an intracellular signaling cascade leading to the binding of the adaptor proteins Tube and Myd88 to the Toll/interleukin-1 receptor (TIR) domains via their death domains (DD) (Sun et al., 2002; Tauszig-Delamasure et al., 2002). Together with the kinase Pelle, Tube and Myd88 form a heterotrimeric complex (Valanne et al., 2011; Towb et al., 2001; Belvin and Anderson, 1996). The Toll signal can be enhanced by the binding of positive regulator Pellino to Pelle (Haghayeghi et al.). The signal from the Myd88-Tube-Pelle complex leads to the phosphorylation and eventual degeneration.
of the inhibitor protein Cactus (Geisler et al., 1992; Kidd, 1992). In absence of a stimulus Cactus binds to the NF-κB transcription factor Dorsal and Dorsal-related-immune-factor (Dif) (Rutschmann et al., 2000) preventing their translocalization to the nucleus (Wu and Anderson, 1998). The phosphorylation of Cactus, that is required for the release of Dorsal/Dif, may be executed by Pelle or yet another unknown kinase (Towb et al., 2001). After their release Dorsal/Dif pass the nuclear membrane to bind NF-κB related binding motives of many genes including the one coding for antimicrobial peptide Drosomycin (Reichhart et al., 1993; Steward, 1987; Ip et al., 1993; Manfruelli et al., 1999).

**Immune deficiency (IMD) pathway**

Like in the Toll pathway pathogen recognition in the *Drosophila* IMD pathway is carried out by PGRPs, namely PGRP-LC (or Ird7) and PGRP-LE (Wu et al., 2001; Kaneko et al., 2006; Choe et al., 2005). The type-2 transmembrane receptor PGRP-LC and the cytoplasmic PGRP-LE can bind directly to meso-diaminopimelic acid (DAP)-type peptidoglycans present in the cell walls of many Gram-positive bacteria (Lim et al., 2006). The death domain containing protein Imd can bind to both PGRP-LE and PGRP-LC, as well as to FADD (Fas-associated death domain protein) (Georgel et al., 2001; Naitza et al., 2002).

How exactly the interaction of these proteins contributes to the activation of immune signaling pathway is still an open question (Kounatidis and Ligoxygakis, 2012). Another factor that is recruited to the IMD protein complex is DREDD. This *Drosophila* homolog of mammalian caspase-8 (Chen et al., 1998; Leulier et al., 2000) processes IMD, thereby revealing a protein domain to which the *Drosophila* Inhibitor of apoptosis-2 (IAP-2) binds (Paquette et al., 2010). By ubiquinating and stabilizing the IMD protein complex IAP-2 supports the recruitment of yet more downstream components such as transforming growth factor activated kinase 1 (TAK1) (Vidal et al., 2001) and its adaptor TAK1-binding proteins 2 and 3 (Kleino et al., 2005). The final steps in this signaling pathway include the activation of the IκB-Kinase complex containing the products of the genes *ird5* and *kenny* through TAK1 causing the phosphorylation of a NF-κB domain containing protein called Relish (Dushay et al., 1996; Hedengren et al., 1999). However, before Relish can translocate into the nucleus it has to be cleaved to relieve it from its C-terminal inhibitory ankyrin repeats, which are similar to the IκB/Cactus domain (Stöven et al., 2003). This
Proteolytic cleavage is likely performed by the DREDD (Stöven et al., 2003; Silverman et al., 2000; Ertürk-Hasdemir et al., 2009).

**Melanization cascade and blood clotting**

Melanin is believed to be important to prevent loss of blood through wound sides, or for directly killing certain pathogens by its toxic properties (Cerenius and Söderhäll, 2004). It is the final product of a proteolytic cascade mediated by melanization protease MP1 and MP2 (Wang et al., 2014). Ultimately their activity leads to the cleavage of prophenoloxidase into its active form phenoloxidase (PO). Crystal cells express two zymogens of PO (DoxA1 and CG8193) and lamellocytes contain a third. When the active form is present in the hemolymph melanin is also deposited on the eggs of parasitic wasps (Ashida and Brey, 1997). The production of melanin can be inhibited by Serpin-27A through its binding to an unknown effector target (Ligoxygakis et al., 2002; De Gregorio et al., 2002). Because of its importance for closure of wounds inflicted by i.e. septic injuries to the cuticle, some considers PO dependent immunity to be the earliest and most acute response (Kounatidis and Ligoxygakis, 2012). However, a direct link between pathogen recognition and activation of the PO based immune response still awaits its identification (Kounatidis and Ligoxygakis, 2012).

Another way in which pathogens are insulated in *Drosophila* is by blood clotting. Besides preventing the loss of blood, the main purpose of coagulation is to inhibit the dispersal of infectious agents from the side of injury (Sun, 2006). For a successful clot formation several proteins are required (Karlsson et al., 2004). Besides Hemolectin that is produced by plasmatocytes, *Drosophila* blood clots also contain proteins of humoral origin such as Lipophorin, Hexamerin and its receptor called Fat Body Protein 1. This demonstrates the importance of collaboration between factors produced by the different arms of the immune defense. The production of another protein involved in the early formation of the clot called Fondue has shown to be directly controlled by the Toll signaling pathway (Scherfer et al., 2006). Although blood clotting was also reported to occur in proPO mutants (Scherfer et al., 2004), the fact that blood clots from larvae devoid of crystal cells fail to harden indicates a direct link between PO and blood coagulation (Bidla et al., 2005). The animals lacking crystal cells were also more susceptible to infections and showed compromised wound healing.
**Vesicular trafficking**

In mammalian cells the innate immune response against certain pathogens involves the activation of the autophagy pathway (Gomes and Dikic, 2014). In *Drosophila* it has been shown that recognition of *Listeria monocytogenes* in the cytoplasm induces autophagy and that this is independent of the Toll and Imd pathways (Yano et al., 2008). Autophagy is best known as a mechanism used by cells under conditions of starvation, in which they utilize their own proteins and organelles as an energy source or as building blocks for new macromolecules. For this, the cargo is non-selectively packaged in double membrane vesicles called autophagosomes, which eventually fuse with lysosomes for their contents to be broken down or recycled (Fig. 10). Also under normal dietary conditions autophagy fulfills a function to maintain cell homeostasis and as a method of waste management (Meléndez and Neufeld, 2008; He and Klionsky, 2009a). Intracellular organelles such as mitochondria or parts of the endoplasmatic reticulum (ER) that are damaged or have accumulated toxins are targeted for destruction and delivered to the lysosomes (Levine and Deretic, 2007).

Figure 10. Main vesicular trafficking events controlled by the vps34-vps15 complex. Adapted from Vanhaesebroeck et al. (2010). Nat Rev Mol Cell Biol. 11(5):329-41
Depending on the context autophagy can promote cell survival or cell death. The latter plays a significant role during developmental processes (Meléndez and Neufeld, 2008). The formation and maturation autophagosomes is a stringently controlled process involving members of different protein families forming a tight regulating network (Mizushima, 2007).

Another process that is traditionally seen as separate from autophagy is known as endosomal trafficking (Fig. 10) (Mulakkal et al., 2014). In order to down-regulate certain signaling pathways, transmembran receptors have to be removed from the plasma membrane by internalization (Vanhaesebroeck et al., 2010). Different proteins associate with the surface of the receptor-containing vesicle forming the early endosome. Through an intermediate stage called multivesicular body (MVB) the vesicle-protein complex matures to form the late endosome, which subsequently fuses to the lysosome.

A third closely related process takes care of extracellular debris or pathogens and is called phagocytosis (Fig. 10). When recognized by the cell, the plasma membrane engulfs extracellular particles by forming a tight ring. When this ring is completely closed the formed sac pinches off from the plasma membrane to be internalized. Depending on its maturation stage and the type of regulating proteins that associate with the vesicle, the formed complex is known as early or late endosome. Despite mechanistic differences and the exact composition of the regulating proteins, the ultimate goal of phagocytes, endocytosis and autophagy is the same: to deliver cargo to the lyosome for degradation or recycling (Vanhaesebroeck et al., 2010).

**Autophagy**

Many genes that regulate autophagy have been originally identified in yeast in the course of genetic screens performed some 30 years ago (Thumm et al., 1994; Tsukada and Ohsumi, 1993; Harding et al., 1995). Commonly known as autophagy related genes (Atg) (Klionsky et al., 2003), their protein products can assemble into complex structures serving as regulators and scaffolds for the formation of autophagosomes in yeast, *Drosophila* and mammals (Mizushima et al., 2011). In all eukaryotes Atg1 is usually considered to be the protein functioning most upstream in the autophagy pathway. This serine/threonine kinase binds to Atg13 (Chang and Neufeld, 2009) and receives input from the insulin/TOR pathway, thereby translating information about nutrient status and energy levels (He and Klionsky, 2009b). Atg101 and the scaffolding protein Atg17 join the Atg1 containing protein
complex to participate in autophagosome formation (Hosokawa et al., 2009). Starvation cause Atg13 to be dephosphorylated leading to the induction of the autophagy pathway by Atg1 (Neufeld and Baehrecke, 2008). Alternatively a protein complex containing the Atg proteins 2 and 18 relays developmental signals such as increased ecdyson titers thereby activating autophagy in doomed cells.

Membranes of autophagosomes, endosomes and phagosomes all contain lipids called phosphatidylinositol-3-phosphate (PI3P) that are the product of a highly conserved kinase complex (Fig. 10) (Lindmo and Stenmark, 2006). In all three major vesicle transport processes, autophagy, endocytosis and phagocytosis the core of this protein complex is formed by the catalytically active class III kinase Vps34 (PI3KIII) and its regulatory subunit Vps15 (Lindmo and Stenmark, 2006). Both were given their name because mutations in these genes affect vacuolar protein sorting in yeast. In Drosophila vps15 is known as immune response deficient 1 (ird1) and was discovered in a screen searching for genes regulating the expression of Drosomycin in response to a bacterial challenge (Wu et al., 2001). When functioning in autophagy, the Vps34-Vps15 kinase complex is dependent on being bound to Atg6 (Beclin1 in mammals) and Atg14 (Juhász et al., 2008; Lórincz et al., 2014). The activity of this complex can also be directly influenced by ecdyson titers (Rusten et al., 2004). Apart from Ird1 another WD40 repeat containing phospholipid effector that is recruited to Vps34 is Atg18 (Scott et al., 2004; Wu et al., 2007). Together, this protein complex is thought to promote early steps of autophagy like the formation (nucleation) of the phagophore around the cargo that is targeted for destruction (Zirin and Perrimon, 2010; Vanhaesebroeck et al., 2010; Juhász et al., 2008). Which membrane compartments (i.e. ER or mitochondria) contribute to the formation of the double membrane of the autophagosomes that derives from the phagophores is still an open question (Mulakkal et al., 2014).

The expansion of the autophagosomes membrane depends on the actions of two ubiquitin-like protein conjugation systems involving Atg8 and Atg5-Atg12 proteins (Zirin and Perrimon, 2010). The Drosophila orthologs of Atg8, Atg8a/Atg8b is commonly used to follow the progression of autophagy in vivo. Expression of the ubiquitin-like coat protein Atg8a can be visualized by fluorescent proteins, that emit differently colored light depending on if Atg8a is present in early autophagosomes or late autolysosomes (Rahman et al., 2012; Nezis et al., 2010). Although additional members of the network have been shown to be important for autophagy in the fly (such as Atg9 and Atg2 (Nagy et al., 2014)), the picture of how and when these proteins interact to coordinate the progression and maturation of
vesicles is far from complete (Mulakkal et al., 2014; Zirin and Perrimon, 2010). The accumulation of autophagosomes when MVB formation is genetically blocked suggests that the final step of autophagy, the fusion with the lysosome, depends on interactions with the endocytosis pathway (Juhász et al., 2008; Rusten et al., 2007). Fusion of autophagosome to the lysosome is supported by components of the homotypic fusion and protein-sorting (HOPS) complex. Two of its components Vps18 and Vps38 are known in Drosophila as Deep orange and Carnation and have been shown to also regulate the endocytosis pathway (reviewed in Akbar et al., 2011). The dramatic decrease in pH levels detected when autophagosomes and lysosomes fuse is believed to support the degradation of the cargo by hydrolases. The products of this process are then reintroduced into the cytosol to be used for energy or synthesis of new organelles and macromolecules.

**Endocytosis**

Many genes known as regulators of the endocytic pathway, when mutagenized in Drosophila give rise to tumor like tissue growth in larval and/or adult organs (Vaccari and Bilder, 2009). To maintain homeostasis of transmembrane surface proteins such as receptors they have to be internalized by the formation of membrane sacs (Fig. 10). When the sac closes the resulting vesicle butts off from the plasma membrane and fuses with others to form early endosomes. Rab5, a member of the large family of small GTPases, regulates this process. First identified as ras-like genes in rat brains (reviewed in Zhang et al., 2007), Rab proteins are known to orchestrate the trafficking of vesicles between different membrane compartments such as the recycling endosome and the trans-Golgi network (Pfeffer). In mammalian cell lines, GTP bound Rab5 interacts with Vps15 thereby increasing PI3P production on the early endosome (Murray et al., 2002). Ubiquitinated receptors within the early endosome can be recycled back to the membrane surface, or transported by MVB to the lysosome for degradation (Spang, 2009). The latter process depends on effector molecules such as hepatocyte growth factor regulated tyrosine kinase substrate (Hrs) and early endosome antigen 1 (EEA1) and the proteins of the endosomal sorting complex required for transportation complex (ESCRT) (Simonsen and Tooze, 2009). As an molecular timer for this process Rab5 is replaced by Rab7 (Poteryaev et al., 2010). Apart from effecting endocytosis, mutating any of the ESCRT components (that in Drosophila include vps25, vps28 and vps4) causes a block of autophagy in ovarian follicle cells and in the fat body (Juhász et al., 2008; Rusten et al., 2007). This underlines the above-mentioned notion that endocytosis and autophagy are
closely interconnected. Similarly to autophagosomes, the fusion of the MVB with the lysosome (marked by arrival of the lysosomal-associated membrane protein 1 (LAMP1) causes the acidification dependent-degradation of its freight (Saksena et al., 2007; Vanhaesebroeck et al., 2010).

**Phagocytosis**

As a specialized form of endocytosis, the basic mechanism of phagocytosis may have evolved from the engulfment of nutrients that can still be observed in single cell organisms (Fig. 10). The first step is to distinguish between self and non-self, which is executed by free and cell bound PRRs in the hemolymph. Apart from PGRP-LC and PGRP-SC1 (Garver et al., 2006), these proteins include *Drosophila* Scavenger receptors like Croquemort, PDGF- and VEGF-receptor related (Pvr) (Wood et al., 2006), Down syndrome cell adhesion molecule (Dscam) and EGF-like repeat-containing proteins like Nimrod and Eater (reviewed in Ulvila et al., 2011). The recognition activates signaling cascades within the phagocytes that cause a remodeling of actin cytoskeleton. This leads to an actin-driven engulfment of the foreign particles in nascent phagosomes that in *C. elegans* and mammalian cells seem to directly interact with the Vps34-Vps15 complex and GTPase Dynamin (Shibire in *Drosophila*) (Vanhaesebroeck et al., 2010). Only then is a GTP-bound Rab5 recruited to the complex, which regulates the maturation of the phagosome until its fusion with the lysosome when Rab7 replaces it (Bucci et al., 2000; Harrison et al., 2003). Like in autophagy and endocytosis, the digestion of microorganisms in the phagocytosis pathway relies on acidification of the vesicle when fused to the lysosome. This process is inhibited in plasmatocytes obtained from flies that are deficient for the full-of-bacteria or the psidin gene (Akbar et al., 2011; Brennan et al., 2007).
**PAPER I** (manuscript)

**Introduction to the screen**

Our lab and others could previously demonstrate that the regular banded pattern formed by sessile blood cells in the *Drosophila* third instar larva is missing in animals that have been infected by the parasitic wasp *L. boulardi* (Zettervall et al., 2004). This was visualized by driving the expression of GFP with the promotor for the hemocyte specific gene *Hemese (He)* using the binary UAS/GAL4 system (Brand and Perrimon, 1993). In addition, this blood cell mobilization phenotype could be mimicked by cell specific expression of certain genetic constructs, including one coding for a constitutively active form of Tl receptor called UAS-Tl\textsuperscript{10b} (paper III, Fig. 7 C and Zettervall et al., 2004). Larvae carrying the corresponding gain-of-function allele Tl\textsuperscript{10b} have previously been described to show certain signs of an activated cellular immune system such as an increased population of circulating blood cells and the formation of lamellocytes (Qiu et al., 1998; Lanot et al., 2001). Also it has been known that under conditions of constitutive Toll signaling blood cell can clump together and melanize in so called nodules or psuedotumors (Minakhina and Steward, 2006). In a more recent study, we could add abnormal lymph gland morphology and an irregular sessile hemocyte-banding pattern to the list of Tl\textsuperscript{10b} phenotypes (paper II).

The ability to rapidly carry out unbiased genome-wide screens for pathway components by identifying loci that modify specific phenotypes has proven to be a particularly powerful
method when Drosophila is used as a model system (St Johnston, 2002). In this approach, a sensitized genetic background exhibiting an easily scored phenotype is used in search genes that when mutated can make this phenotype more severe (enhancer) or more like wild type (suppressor) (Ryder et al., 2004; Bier, 2005).

In the present study (paper I) we conducted a genetic modifier screen of the right arm of the third chromosome. Our goal was to identify genes that have so far not been implemented to have a function in cellular immunity downstream of Toll (Fig. 11). The sensitized genetic background was provided by the Tl10b allele and the sessile hemocyte mobilization phenotype was visualized by the blood cell-specific driver HmlΔ-Gal4 expressing UAS-GFP (shorthand: HmlΔGFP) (Sinenko and Mathey-Prevot, 2004). Into this genetic background, additional mutations were introduced by crossing HmlΔGFP; Tl10b males to females obtained from a collection of fly stocks each carrying a single heterozygote deletion (also known as deficiency) of a part of the fly genome. We used the DrosDel collection of deficiency lines (Ryder et al., 2004), carrying deletions in the right arm of the third chromosome (3R). Created in a similar isogenetic background, an additional 14 deletion lines from the Exelixis collection (Parks et al., 2004) were later included in our study in order to achieve higher coverage for certain genomic region of interest, or to substitute for DrosDel strains not viable when their balancer chromosomes were exchanged for our purposes.

**Practical aspects of the genetic screen**

Initial tests on the robustness of the Tl10b loss-of-sessile band phenotype revealed that temperatures below 25°C could (to a certain degree) suppress blood cell mobilization. In contrast, Tl10b larvae sampled from a crowded culture showed in general a more severe phenotype. In addition, I noticed that the penetrance of the loss-of-sessile band phenotype could differ between larvae emerging early from the culture and those that crawled out of the food last. The correct staging of the larva used for our assays was also of major importance. With the onset of pupariation all cells are lost from the sessile compartment even in wild-type individuals. During the cause of these pilot experiments it was also noticed that the Tl10b allele (but not other marker mutations) could sometimes get lost in a fly culture. If males from this stock were used for the crosses, the larvae from a “Tl10b” mutant control cross would very much resemble those of the wild-type control cross. Lastly, the suppressive effect that could be achieved by crossing known loss-of-function mutants of Tl pathway mediators to Tl10b flies seemed to depend on the age of the parents. While almost all
larvae produced by these flies in the beginning of the experiment showed a rescued sessile hemocyte pattern, this effect was less prevalent as the parents reached an age of more than 2 weeks (data not shown).

To account for these confounding factors we established a protocol that was stringently followed during the course of this study. Males and females were collected right after eclosure and always inspected for having the correct marker mutations before being used for the crosses. In particular, we used only $Tl^{rob}$ males that showed at least one clear nodule in their body. By this we wanted to ensure that the $Tl^{rob}$ allele was still present in all individuals used. Before mating, males and females were kept separately from each other for up to four days. By this we wanted to enhance their eagerness to mate and ensure that all females were in the optimal age for egg laying (Ashburner et al., 2005). To avoid crowded conditions, all fly crosses were performed using about the same ratio of males to females (5/20) in bottles containing sufficient food. The fly cultures were kept in an incubator at 25°C and 60% humidity and parents were transferred to new bottles every two days. To ensure that no offspring was analyzed that originated from too old parents adult flies were discarded from the bottles after a maximum of ten days.

To control for other unknown external factors that could have influenced the outcome, two control crosses were always conducted in parallel with each set of experimental crosses. For these, females having the same genetic background as the deletion stocks but being otherwise wild type were crossed to either $Hml^P$-GFP; $Tl^{rob}$ males (mutant control) or to males carrying $Hml^P$GFP alone (driver or wild-type control). To ensure that all larvae collected from these cultures were at a similar stage of development and not already undergoing pupariation, we relied on several parameters. Firstly, only larvae of the wandering stage were collected that had left the food in search for a place to form the pupa. Secondly, it was ensured that the larvae were still crawling and did not show any morphological signs indicative of the onset of pupariation. These include the shortening of the body, the complete eversion of the anterior spiracles, and the tanning and hardening of the larval skin (Bodenstein, 1950, and Ashburner et al., 2005). Lastly, the fly food used for these cultures contained a dye that makes it visible in the intestine of the semitransparent larva. It was ensured that only larvae were collected for analysis that had not already emptied their gut contents completely, which is another sure sign for the imminent onset of pupariation (Andres and Thummel, 1994). To stall further development between the collection and analysis of the larvae they were kept in water filled petri dishes on ice. For microscopy they were transferred on to glass slide kept on ice-cold aluminum.
plates, submerged by glycerol and mounted by a cover slip. To further immobilize the larvae before inspection the glass slides were incubated for 20 min at -20°C or over night at 4°C.

Each experiment conducted in this study was comprised of at least five different crosses of $Hml^D$GFP-$Tl^{10b}$ males to females collected from as many stocks carrying deficiencies or other mutations (experimental crosses), as well as the two control crosses. To be able to quickly evaluate the appearance of the sessile hemocyte pattern in offspring larvae a semi-quantitative scoring method was devised. As published by us earlier (papers II and III), an examiner blinded for the identity of the crosses assigned third instar larvae with individual mobilization grades from 1 to 4. Grade 1 was given to larvae with sessile blood cell patterns resembling those usually found in the wild-type, grade 4 to animals similar to mutant control larvae; grades 2 and 3 were reserved for intermediate phenotypes (see paper I, Fig. 1 A). After every larva sampled for the experiment had been scored, the genotypes of the larva was revealed to the experimenter. An average mobilization grade was calculated from all individual grades given to larvae originating from the same experimental cross and compared to the values calculated for wild-type and $Tl^{10b}$ mutant control larvae. If the average mobilization grades calculated for the control crosses differed substantially from the expected values, the obtained data was not included into the analysis and the entire experiment was repeated.

Deficiencies producing very little progeny when crossed to $Hml^D$-$Tl^{10b}$, or those having either a particularly striking or variable effect on the sessile hemocyte-banding pattern, were included in up to seven independent experiments. To be able to generalize the effect of these deletions, we introduced an indicator called mobilization index (MI) and defined it as the sum of all average disruption grades calculated from all crosses made with flies originating from the same deletion stock, divided by the total number of times the particular deficiency was tested.

**Screening Results/Discussion**

**Classifications of Deficiencies**

Deficiencies that scored with an MI that was within the range of the wild-type control crosses (MI =1.66, SD=0.25) were defined as weak or strong suppressors (paper I, Fig. 1 B). Of the 88 deficiencies tested, 17 were categorized as weak and 13 as strong suppressors (paper I, Table S1). In contrast, as none of the deficiencies
tested had an MI that was substantially larger than the one calculated for the $T^{rob}$ mutant control crosses ($MI = 3.21, SD=0.36$), no enhancers could be determined. As all deficiencies used for our study unambiguously map to the fly genome with single base pair resolution (Parks et al., 2004; Ryder et al., 2004), we could plot calculated M.I. values against their length and chromosomal positions (paper I, Fig. 1 C). Besides the fact that we had achieved about 90% coverage of the chromosome arm, this depiction also showed that the deficiencies deemed by us as strong suppressors fell into five separate genetic regions (paper I, highlighted in red in Fig. 1 C).

In general the distribution curves for the frequency of M.I. values calculated for crosses to deficiency stocks, wild-type and $T^{rob}$ mutant control crosses were skewed towards the lower range of the scale (paper I, Fig. 1 B). There are several possible explanations for this trend. Firstly, although the examiner was blinded for the origin of the larvae during the assay it is conceivable that subjectivity may have influenced the results. The introduction of a deficiency may be conceived by her/him as a form of intervention that is expected to make a difference to the phenotype. However, the $T^{rob}$ allele by itself already causes a strong disturbance in the sessile hemocyte-banding pattern. It is therefore possible that only very few deficiencies are actually able to enhance this phenotype. Therefore, rather than ruling that the intervention had no effect on the phenotype, the examiner may have in general unconsciously allotted too small grades to the inspected larvae.

Deficiencies causing lethality

About 10% of the tested deletion caused lethality when being placed as homologs to the $T^{rob}$ allele-bearing chromosome (paper I, Table S1). Unspecific effects generated by the change in genetic background when the $T^{rob}$ allele chromosome replaced the balancer may be one explanation. Alternatively given the fact that the $T^{rob}$ allele is homozygous lethal by itself (Erdelyi and Szabad, 1989), these deletions could be regarded as enhancers. If so, the chromosomal regions covered by these deletions may contain genes functioning as negative regulators of the Toll signal. By using lethality in $T^{rob}$ genetic background as readout, additional deletions and mutations could be tested in the future to uncover the identity of these genes.
Internal controls

We had two main reasons for focusing our efforts on 3R. Firstly, due to the comparatively high number of deletions that was available for this genomic region at the time, we expected to achieve a high coverage. In fact, as many deletions have overlapping breakpoints, we were able to cover around 90% of 3R with a minimal subset of 88 deletions. Secondly, many important members of the Toll signaling pathway, including tube, pelle, Dif, dorsal, and Toll itself are situated on this chromosome arm. Therefore, we expected that deletions covering one or more of these genes could serve as internal controls. However, neither the deletions covering tube, pelle nor Tl/spz/pll fell into the categories set by us for weak or strong suppressors (paper I, Fig. 1 C and, Table S1). This may simply indicate that a theoretical reduction in dosage by 50% for these genes is insufficient to affect the Tl10b phenotype. In fact, there is evidence that the real reduction of gene dosage that can be achieved by heterozygous deletions is only around 44% (Stenberg et al., 2009). This is due to a regulating process called functional dosage compensation or buffering effect (Stenberg et al., 2009). Thereby i.e. the presence of three copies of the same gene does not necessarily cause a 3-fold increase in mRNA levels but only a ≈ 1.4 fold increase compared to wild type (Gupta et al., 2006; Zhang and Oliver, 2007; Johansson et al., 2007). However pilot experiments conducted by me earlier showed that most Tl10b larvae heterozygous for Myd88c03888 (Tauszig-Delamasure et al., 2002) or pelle7 (Shelton and Wasserman, 1993) loss-of-function alleles have almost wild-type looking sessile hemocyte banding patterns. One can speculate if these contradictory results may indicate that the buffering effect is stronger when the genome is missing copies of hundreds of genes compared to only carrying mutations for single genes.

headcase (hdc)

Results/discussion

In search for another way to verify the reliability of our screening approach, I decided to test mutant alleles for the gene hdc. Together with three others genes, hdc is covered by the Df6332 categorized by us as a weak suppressor (paper I, Fig. 1 C, Table 1 and Table S1). Of a total of 111 366 bp included in the deletion, hdc occupies 94 450 bp or 85% of this deletion. I was able to replicate the suppressive effect of
Df6332 on the Tl\textsuperscript{rob} blood cell mobilization phenotype with two independent hdc loss-of-function mutants (paper I, S2 A). Importantly, this effect was lost when I expressed a wild-type construct coding for full-length hdc (hdc\textsuperscript{FL}) specifically in the hemocytes of Tl\textsuperscript{rob} larvae heterozygote for one of these alleles. This makes it likely that hdc was responsible for the suppressive effect of the deletion. In addition it indicates that expression of this gene is required specifically in hemocytes for the mobilization of sessile blood cells in response to a constitutive Tl signal. From the four tested UAS-hdc-RNAi constructs only the one from the KK collection (KK 104322) could mimic the suppressive effect of the alleles when expressed with Hml\textsuperscript{Δ}GFP in Tl\textsuperscript{rob} mutant larvae (paper I, Fig. S2 A). Fly lines originating from the GD library had either no effect (GD39876 and GD45069) or caused lethality (GD39877, see below). Coherently the KK construct was most efficient in silencing hdc expression in hemocytes (paper I, Fig. S2 B).

However, there is an alterative explanation for the different results obtained with GD and KK fly lines. The KK fly stock library was created by the Vienna Drosophila RNAi center in order to overcome shortcomings of their older GD collection (Dietzl et al., 2007). The short hairpin RNAs (shRNAs) carried by GD fly stocks were integrated into the Drosophila genome more or less randomly by P-element mediated transformation. Thereby they could create false positives by disrupting the function of an endogenous gene, or false negatives by integrating into transcriptionally silent regions like heterochromatin (Green et al., 2014). In contrast, by using the pKC43 vector in a two-step transformation process, the shRNAs for the KK library should always integrate themselves at the exact same spot of the Drosophila genome (Green et al., 2014; Groth et al., 2004). However, recently it was reported that the genetic background of the KK stocks is less homogeneous than anticipated. While some KK stocks carry only one shRNA copy others carry two (Green et al., 2014). Possibly more important, one of the integration sides of shRNAs is within the 5’ untranslated region of tiptop. This gene codes for a protein belonging to the highly conserved Teashirt family of transcription factors, known to play a role in the differentiation of certain nephrocytic cells in Drosophila (Denholm et al., 2013). This may explain why some of the KK lines produce phenotypes such as wing defects or pupal lethality when crossed to driver lines that cannot be explained by the particular shRNA construct they carry (Green et al., 2014). Therefore, at this point I cannot rule out the possibility that it is the disruption of tiptop gene function not the silencing of hdc gene expression that is responsible for the suppressive effect of the KK line on our phenotype. If this could be excluded by further test, then results
obtained with the RNAi line KK104322 can be taken as support for the hypothesis that hdc fulfills a crucial role in blood cell mobilization in response to Tl signaling.

The gene hdc was named after the fact that null mutant flies are lethal as they fail to properly form a head capsule during metamorphosis (Weaver and White, 1995). Silencing gene function by UAS/GAL4 regulated RNAi construct expression is usually not expected to give stronger phenotypes than observed in mutant nulls (Dietzl et al., 2007). It is therefore surprising that when crossing the line GD39877 to HmlΔGFP; Tl^rob, I repeatedly failed to obtain any offspring beyond embryonic stage (paper I, suppl. Fig. 2 A). Even more unexpected was the finding that even in absence of the Tl^rob allele, crossing this hdc-RNAi construct to the HmlΔGFP driver line produced very few larval offspring. In fact none of the three independent crosses conducted yielded the 10 larvae that we required for analyzing hdc-mRNA levels in pooled blood samples by Q-RT-PCR (paper I, Fig. S2 B and data not shown). As with the KK line, the cause of this may not be the silencing of hdc expression but the genetic background of this particular hdc-RNAi line. Else wise, the fact hdc-RNAi line GD39877 causes lethality when driven with HmlΔGFP could be taken as evidence that hdc expression in hemocytes is not only important for Tl signaling phenotypes but in general for Drosophila development.

Further literature reflections

The developmental gene hdc was the first to be described being expressed in all imaginal tissues (Weaver and White, 1995). These include the ovary, imaginal discs of leg, eye, wing and haltere, as well as scattered cells of the hindgut, midgut and foregut (Weaver and White, 1995). Unlike larval cells, changes in ecdyson titers induce the re-entering of imaginal cells into the mitotic cycle at time points that can vary between the different imaginal primordial. The proliferation and patterning of these cell sacs is a strictly regulated program that when altered can lead to aberrant body structures in the adult (Weaver and White, 1995). As hdc expression peaks 24h before the re-entry of imaginal cells it is believed to regulate proliferation. However, shape and size of imaginal discs are normal in hdc null mutants despite the fact that they all die as pupae (Weaver and White, 1995).

Most importantly for our study, expression of hdc has previously been shown in the lymph gland but not in circulating hemocytes (Weaver and White, 1995; Márikus et al., 2009). I could confirm these results by using an Hdc antibody (Weaver and White, 1995) and by enhancer trap lines in which hdc expression is coupled to lacZ (Steneberg et al., 1998) or GFP (data not shown). In a previous study it was
reported by us that the hematopoietic defects of Tl10b mutant larvae also extent to the lymph gland (paper II). It is therefore conceivable that hdc expression in this hematopoietic organ could also be important for communication between cells within and outside the lymph gland. Although this may explain or finding that hdc suppresses Tl10b loss of sessile cells, so far it has not been shown that the i.e. the posterior signaling center exerts a control beyond the adjacent medulary zone in the lymph gland. hdc expression has however previously been shown to be important in certain imaginal fusion cells to suppress neighboring cells from growing aberrant tracheal sprouts in a cell non-autonomous manner (Steneberg et al., 1998).

Recently it has been reported that in specific somatic cells of the Drosophila testis called hub cells hdc expression is required to prohibit their programmed cell death (Resende et al., 2013a). Hub cells can be found at the tip of the testis where they are crucial for the maintenance of germline and cyst stem cells. Signaling pathways such as JAK/STAT and Hedgehog, control the function and morphology of the testis stem cell niche in a similar fashion as in the Drosophila lymph gland (Resende et al., 2013a; Krzemien et al., 2010). Also in the developing Drosophila eye hdc has previously been shown to act as a negative regulator of STAT transcription factor Stat92E (Bach et al., 2003). Consequently, Resende and coworkers could significantly reduce the number of hub cells by expressing hdc-RNAi constructs with a GAL4 driver line controlled by JAK/STAT ligand unpaired (Upd). The strongest effect they achieved by using the GD45069 line, whereas GD39877 and KK104322 only slightly reduced the number of hub cells.

In my hands, driving hdc-RNAi with HmlΔGFP in Tl10b or wild-type genetic background had the strongest effect when the KK104322 line was used, whereas GD45069 had no effect and GD39877 caused lethality. As stated above, this discrepancy could be explained with differences in the genetic background of these stocks. Alternatively, the partially contradictive effects that these hdc-RNAi carrying stocks had on blood cell and testis phenotypes may arise from the importance of the different hdc isoforms. It has been claimed that the 104322KK stock is the only one carrying an hdc-RNAi construct capable of targeting all three hdc mRNA isoforms (Resende et al., 2013b). It is possible that for the maintenance of hub cells all isoforms are equally important whereas the third isoform plays the major role in the lymph gland. Establishing a link between hdc and immunity would help to explain growing evidence connecting its human homolog to carcinogenesis (Dowejko et al., 2012; Makino et al., 2001; Chien et al., 2006).
**Rab23**

**Results/Discussion**

With the help of additional deficiency lines, suppressive region 1 was mapped down from originally including almost 360-kilo base pairs (kb) to just 30 kb. Its suppressive nature could be independently confirmed by a deletion (Df7283EX) originally created for the Exelixis deficiency kit (paper I, Fig. S1 A). Due to time constrains, only one out of seven genes included in region one was tested (paper I, Table 1). Coding for a members of the small GTPase protein family, Rab23 is mostly known for being a negative regulator of Hedgehog (Hh) signaling in mouse development (Eggenschwiler et al., 2006). I obtained the alleles Rab23<sup>T69A</sup> and Rab23<sup>S1</sup>, both previously described to behave as strong-loss-of-function or null alleles of Rab23 (Pataki et al., 2010). When crossed independently of each other to Hml<sup>Δ1-Tl<sup>10b</sup></sup>, both alleles produced larvae whose disruption grades were significantly lower compared to the mutant control crosses (paper I, Fig. S1 B). However Df5196DD (a deletion that fell just outside our category for weak suppressors) covers the transcription start side of Rab23 (paper I, Fig. S1 A). Therefore, it cannot be excluded that the gene responsible for the strong suppressive effect of region 1 may not be Rab23 but one or more of the other genes situated in its proximity.

**Further literature reflections**

Like other small guanosine triphosphatase (GTPases), Rabs change their conformation depending on if they are bound to GTP or GDP. The binding of GTP (essential for the interaction of Rab proteins with their target proteins) is initiated by guanine nucleotide exchange factors in response to certain stimuli. Cessation of the interaction is achieved by the hydrolysis of GTP to GDP, which is promoted by GTPase-activating proteins (Citalan-Madrid et al., 2013). As a member of this family of proteins regulating vesicular trafficking, Rab23 is believed to transport components of the Hh pathway and function between Smoothened and transcription factor Gli2 (reviewed in Fuller et al., 2014). This would explain why mice deficient for Rab23 show a phenotype known as open brain that is consistent with the known effect of constitutive Hh signalling, including severe neural tube malformation, poor eye sight and polydactyli (Günther et al., 1994; Eggenschwiler et al., 2001). Interestingly like the membrane receptor EGFR, Rab23 is one of few cytosolic proteins that can also be found in the nucleus in certain cancer cells (Huang et al.,
Apart from its role in cancerogenesis, an unusual splice variant caused by a mutation of Rab23 has been shown to be involved in the development of the Carpenter Syndrome (Ben-Salem et al., 2012).

In the *Drosophila* lymph gland expression of Hh signaling molecules is restricted to the posterior signaling center (PSC), whereas the pro-hemocytes of the adjacent medullary zone (MZ) express the corresponding receptor Patched and transcriptional effector cubitus interruptus (Ci). The communication between these two subsections of the lymph gland is believed to be insured by filopodia that stretch from the PSC into the MZ (Lebestky et al., 2003; Krzemien et al., 2007; Mandal et al., 2007). The expression of the Hh signaling molecule in the PSC depends on GATA factor Serpent. To prevent its expression in the pro-hemocytes of the MZ and the differentiated blood cells of the cortical zone (CZ) the lymph gland depends on transcriptional regulators U-shaped and Suppressor of Hairless (Tokusumi et al., 2009). These factors are also involved in the differentiation of embryonic blood cells that form the reservoir for the different hemocytes types that are found in the larva (Evans et al., 2014). If the molecular events involved in the Hh pathway are distorted in the lymph gland, the blood cells of the MZ undergo premature differentiation into plasmatocytes and crystal cells causing an aberrant morphology of the organ (Mandal et al., 2007; Tokusumi et al., 2012). If it were to be shown that the Hh pathway also controls differentiation of sessile and circulating blood cells, its perturbation may very well cause modifications of *Tl* larvae hematopoietic phenotypes observed by me. As Rab23 is believed to be a negative regulator of Hh, loss-of-function mutations should enhance Hh signaling in the entire larva. In theory this may hinder peripheral blood cells to differentiate into full-fledged plasmatocytes capable of responding to an immune stimulus. If this cell type would further populate the sessile compartments it could explain my finding that *Tl* larvae heterozygote for Rab23 alleles retain their sessile hemocyte banding pattern. The JAK/STAT pathway has been shown to fulfill a similar conservation role in the lymph gland as Hh. However mutation rendering this pathway constitutively active have been known to cause hemocyte activation phenotypes such as nodule and lamellocyte formation in peripheral blood cell population (Hou et al., 1996; Yan et al., 1996; Müller et al., 2005). The fact that JAK/STAT has the opposite effects outside compared to inside the lymph gland calls for some caution when it comes to predicting the effects of signaling pathways in different tissues.

Rab proteins have been shown to be crucial regulators of autophagy (Chua et al., 2011). Besides its established role in degradation and recycling of cell native
molecules and organelles, evidence is accumulating that autophagy also plays a key role in defence against bacterial infections. Recently it has been shown that in mammalian HeLa cells Rab23 associates with Atg5 positive isolation membranes shortly after S. pyogenes infection (Nozawa et al., 2012). In contrast to Atg5 however, Rab23 remained associated with S. pyogenes containing autophagosomes as they matured to lysosomal-associated membrane protein 1 (Lamp1) positive autolysosomes that commit their cargo to destruction. Knockdown of Rab23 hindered formation of S. pyogenes containing autophagosomes. However, autophagosomes that were formed as a response to starvation did not recruit Rab23, indicating that this gene functions exclusively in the immune response. Like for autophagy also for the development of many Hh dependent human cancers, PI3K plays a central role. Specifically pancreatic cancers show a high incidence of PTEN loss of function mutations known cause overactivation of insulin-like growth factor/PI3K/Akt pathway (Riobo et al., 2006). This notion that autophagy and its mediators may play an important role besides just initiating starvation reactions was further strengthened by work on another candidate gene identified in our screen called ird1.

**pumilio (pum)/D1 chromosomal protein (D1)**

**Results/Discussion**

Strong suppressor regions 2 could be mapped down from 318 kb to just 18 kb including only eight genes (paper I, Fig. S1 C and Table 1). To test the translational repressor gene pumilio, two homozygous lethal alleles pum3 and pum13 were crossed to HmlΔGFP; Tl10b, the latter being a known loss-of-function mutation (Tearle and Nüsslein-Völlhard, 1987; Wharton et al., 1998; Sonoda and Wharton, 2001). Both failed to replicate the suppressive effect of the smallest deficiency tested covering this region (Df6152EX). Instead, they produced offspring that was scored with similar disruption grades as mutant control larvae (paper I, Fig. S1 D). Consistent with its role as a transcriptional repressor, I did however observed an increased number of individuals showing at least one nodule in larval offspring produced by crosses of Tl10b to either of pum allele bearing stocks (data not shown).

Similarly, a hypomorphic allele of chromosomal protein encoding gene D1 (D1EP473Rec#70), as well as two small deletions covering either D1 alone (Df(3R)D1c12w-) or both D1 and neighboring gene ird1 (Df(3R)D14A), produced on average more larvae
showing at least one nodule compared to \( T^{lohi} \) mutant controls (data not shown). In contrast to the tested \( pum \) alleles however, \( D1 \) alleles also significantly suppressed the loss of sessile band phenotype in \( T^{lohi} \) larvae (paper I, Fig. S1 D). All of these alleles have been created by the mobilization of a \( P \)-element (\( D1^{EP473} \)) (Weiler and Chatterjee, 2009), which had been inserted into the 5'-untranslated region of \( D1 \) in a previous study (Rørth et al., 1998). Precise excision lines created for the same study by re-mobilizing the \( P \)-element of the hypomorph (\( Df(3R)D1^{70-1} \)), or the original \( P \)-insertion stock (\( D1^{Rev1B} \)), also caused a significant suppression of our phenotype. Besides excluding the \( P \)-element itself as a cause for this effect, the result obtained with \( D1^{Rev1B} \) was remarkable because this stock was reported to be wild-type for \( D1 \) (Weiler and Chatterjee, 2009). From this I hypothesized, that the common denominator responsible for the suppression of our phenotype I obtained with each \( D1 \) stock tested may not be the functional ability of the \( D1 \) gene itself, but rather that they all have been created using the same \( P \)-element insertion fly line (\( P\{EP\}D1^{EP473} \)) (Bellen et al., 2004). As this stock was reported to be homozygous lethal (Rørth et al., 1998), it was initially concluded that \( D1 \) function must be disrupted and that the gene itself is crucial for normal embryonic development (Aulner et al., 2002). In a later study however, it was shown that the precise excision of this \( P \)-element did not revert lethality and that the chromosome carrying the insert is in fact viable over the \( Df6152^{EX} \) deletion covering \( D1 \) and other genes (Weiler and Chatterjee, 2009). This indicates the existence of an unknown extraneous lethal mutation carried in the genetic background of the originally created \( P \)-element insertion stock. This mutation is likely responsible for the phenotypes previously attributed to the loss of \( D1 \) by others (Aulner et al., 2002). Also, as all \( D1 \) stocks tested by me derive from the same \( P \)-element insertion fly line, it is possible that the observed modifications of \( T^{lohi} \) blood cell phenotypes were caused by this unknown second side mutation. The notion that loss of \( D1 \) is unlikely to affect \( T^{lohi} \) phenotypes is supported by the findings that not loss of \( D1 \) but over expression of this gene causes ectopic pairing of polytene chromosomes and severe morphological defects leading to lethality (Weiler and Chatterjee, 2009; Smith and Weiler, 2009).
**immune response deficient 1 (ird1)**

**Kinase domain alleles of ird1 modify \( Tl^{10b} \) phenotypes**

Although I could not completely rule out the possibility that loss of \( D1 \) function may still be the cause for the suppressive nature of region 2, I decided to turn my attention to the remaining genes situated in this genomic location. Apart from CG8420 and non-protein coding gene CR45196 (paper I, Table 1), for which no mutant stocks nor information about their molecular function were available, the only remaining gene was immune response deficient 1 (ird1). This gene was originally discovered in genetic screen that used ethyl methanesulfonate (EMS) to uncover mutants that cause late larval stage lethality and inhibit the infection-induced production of the antimicrobial peptide Drosomycin (Wu et al., 2001). Later it could be shown that ird1 is a homolog of vacuolar protein sorting 15 (vps15) from yeast, coding for a serine/threonine kinase with WD40 domains (Wu et al., 2007). The additional alleles created for this study were shown to carry point mutations in the ird1 open reading frame. Besides replicating all phenotypes observed with the original mutant allele, homozygous animals showed constitutive expression of the gene coding for the primary target of the Toll signaling pathway, Drosomycin. This latter phenotype, as well as the spontaneous formation of melanotic capsules reported previously for ird1 null mutants (Wu et al., 2001), is also a known feature of \( Tl^{10b} \) gain-of-function mutants such as \( Tl^{10b} \) (Lanot et al., 2001; Qiu et al., 1998). Although this made it seem unlikely that ird1 is the gene responsible for the suppressive effect on \( Tl^{10b} \) mapped by us to region 2, I decided to test confirmed mutant alleles with our assay.

When tested by me, out of five point mutation alleles only ird1\(^5\) suppressed the loss of sessile cells phenotype when crossed to \( Hml^{\Delta}\)GFP; \( Tl^{10b} \) and substantially increasing the frequency of individuals with nodules (paper I, Fig. 2 B and C). Genetically these alleles differ from each other in the exact position of the mutations (paper I, Fig. 2 A). There are nonsense mutations in the ird1\(^1\), ird1\(^2\), ird1\(^3\) and ird1\(^5\) alleles, causing premature stop of translation, whereas ird1\(^4\) has two amino acids substituted. One of these amino acid changes is highly conserved in evolution. The mutations in ird1\(^3\) and ird1\(^5\) are within the WD40 repeats and the kinase domain. Only ird1\(^5\) carries a stop codon that truncates the protein already within the kinase domain (paper I, Fig. 2 A and Wu et al., 2007). Besides my finding, also when it comes to failing to induce autophagy after starvation ird1\(^5\) has been reported to be the
strongest allele (Lindmo et al., 2008). For the same study a homozygous lethal deletion was created by FLP-mediated DNA mobilization (Parks et al., 2004), lacking the entire \textit{ird1} open reading frame \cite{paper I, Fig. 2 A}. When testing this allele (named \textit{ird1}\textasciitilde{hap} for the present study), I obtained the strongest suppression of the \textit{Tl} \textasciitilde{ob} loss of sessile cells phenotype of all tested \textit{ird1} stocks and a similar increase in the frequency of individuals with nodules like with the kinase domain mutant \cite{paper I, Fig. 2 B and C}.

The original study, for which the EMS mutants were created, used mainly the expression of different antimicrobial peptides as a read out to characterize the different point mutant alleles of \textit{ird1} (Wu et al., 2007). The mutations carrying stop codons in close proximity to each other, \textit{ird1}^t and \textit{ird1}^s, had as homozygotes similar phenotypes when it comes to failing to express Diptericin or Defensin in response to an \textit{E. coli} infection. Like \textit{ird1}^t/\textit{ird1}^s transheterozygotes they also showed a constitutive Drosomycin expression even in absence of an immune challenge. However when the expression of other antimicrobial peptides was measured, the results were much less clear-cut. \textit{ird1}^t null larvae showed low expressions of Attacin, Drosocin and Cecropin after \textit{E. coli} infection compared to controls. In contrast mRNA levels of these antimicrobial peptides in \textit{ird1}^s homozygous animals were either unaffected by the immune stimulus, or even slightly increased compared to wild-type larvae. From these and my results it follows that different parts of the \textit{ird1} protein are critical for different cellular processes. While the kinase seems to be essential for the function of \textit{ird1} as a regulator of cellular immunity and autophagy, the other domains of the protein may fulfill an additional role in regulating certain aspects of the humoral response.

\textit{ird1} loss-of-function activates \textit{Tl} signaling in fat body

In which direction the humoral response is altered in \textit{ird1} mutants seems to depend on the specific pathway (i.e. Toll or IMD) it interacts with (Wu et al., 2007). However, independent of which particular allele/s was used to create \textit{ird1} null larvae in the original study, homogenates from these animals always showed higher \textit{Drosomycin} mRNA levels compared to wild-type controls (Wu et al., 2007). I could confirm this finding using a transgene reporter system, in which the propeptide of Drosomycin is coupled to GFP (\textit{Drs-GFP}). Like larvae heterozygote for \textit{Tl} \textasciitilde{ob}, the majority of \textit{ird1} homozygous or heterozygote animals showed a robust expression of the construct in the fat body \cite{paper I, Fig. 4 A, upper panel}. In contrast to \textit{Tl} \textasciitilde{ob} heterozygotes and animals expressing UAS-\textit{Tl} \textasciitilde{ob} with \textit{Hml}^\Delta\text{GAL4}, neither \textit{ird1} null
nor larvae expressing UAS-ird1-RNAi with various GAL4 drivers showed a Drs-GFP signal in circulating or sessile hemocytes (paper I, Fig. 4 A and suppl. Fig. 3 C). This showed that loss of ird1 expression cannot lead to a constitutive activation of the Toll pathway in the hemocytes. This also indicated that the suppressive effect on the Tl\textsuperscript{rob} loss of sessile cells phenotype that I obtained with some of the ird1 alleles was likely not due to aberrant Tl signaling in the hemocytes. Instead reduced ird1 expression levels may have caused physiological and/or morphological changes in the hemocytes altering their responses to constitutive Toll signaling. My finding that expressing ird1-RNAi in fat body alone by FB-GAL4 is insufficient to activate Drs-GFP gives further supports to this notion (paper I, Fig. S3 C). In fact, ird1 expression needed to be simultaneously silenced in both hemocytes and fat body by Cg-GAL4 to activate Toll signaling in the latter tissue. Previously it has been shown that the increased Drosomycin mRNA levels of ird1 null larvae could be suppressed by loss of spz or Dif (Wu et al., 2007). This points to the possibility that hemocytes depleted of ird1 expression may secrete Spätzle into the hemolymph. When Spz is bound by Tl receptors on the fat body cells this may in turn cause a constitutive production of Drosomycin in this organ. An indirect connection between ird1 and secretion has recently been provided by a study on its binding partner Vps34. Mitotic clone cells deficient for Vps34 in the salivary gland of Drosophila larvae fail to secrete the glue protein required for attachment of the pupa (Shravage et al., 2013). As mutations of Vps34 and ird1 have been shown to give rise to similar phenotypes in the past (Juhász et al. 2008) it would be interesting to test if ird1 loss-of-function could also cause secretion defects. Also, it was recently postulated that genes functioning in autophagy might be directly involved in protein secretion (Deretic et al., 2012)

\textit{ird1} loss-of-function causes plasmatocyte to differentiate into lamellocytes

Our lab and others have previously shown that the constitutive activity of signaling pathways such as JAK/STAT and Tl can cause nodules and lamellocyte formation and an increase of the number of circulating blood cell (Qiu et al., 1998; Lanot et al., 2001; Zettervall et al., 2004; Makki et al., 2010). Relatively recently it became clear that lamellocytes could develop directly from plasmatocytes and that this can take place outside the lymph gland (Honti et al., 2014). A first indication that this could also take place in hemocytes with altered ird1 gene expression levels came from driving ird1-RNAi (Abe et al., 2009) with Hm\textsuperscript{PGFP} in Tl\textsuperscript{rob} larvae. Some of these larvae showed in general very weak or no GFP expression in lymph glands,
sessile or circulating hemocytes (paper I, Fig. 2 B and Fig. 3 A-C). Importantly this effect was independent of Tl signaling, as it could also be achieved by silencing ird1 expression in hemocytes of otherwise wild-type larvae. The particular Hml-GAL4 driver strain used by me was originally created in order to express constitutively active forms of different kinases to activate either Ras/Raf/MAPK or the JAK/STAT pathway in Drosophila larvae (Sinenko and Mathey-Prevot, 2004). This treatment caused the appearance of lamellocytes, which in contrast to most plasmatocytes did not show Hml-GFP expression. From this I inferred that loss of ird1 expression might also force plasmatocytes into the lamellocyte lineage.

Previous it was claimed that an increased number of lamellocytes in Tl10b can only be found in the circulating blood cell population and that their lymph glands show normal morphology (Qiu et al., 1998; Lanot et al., 2001). In contrast, we published recently (paper II), that the Tl10b allele could cause drastic malformations of the lymph gland. This could be confirmed by the present study (paper I, Fig. 3 C and Fig. 5 C). In addition by using blood cell specific antibodies, we could demonstrate that in Tl10b larva lamellocytes can also be detected in various parts of the lymph gland (paper I, Fig. 5 C). In our previous study we could show that only by blocking Tl signaling through Myd88-RNAi expression in the fat body but not in the hemocytes, could we obtain wild-type looking lymph glands in Tl10b larvae (paper II). Also for the development of other known Tl10b phenotypes, such as the increase in circulating blood cell number and nodule formation, Toll signaling in hemocytes was dispensable. In contrast, in the present study it was sufficient to express ird1-RNAi with HmlΔGAL4 to rescue the malformation of lymph glands in Tl10b larvae (paper I, Fig. 3 C). However, the fact that these animals also showed an increase in circulating hemocyte numbers (paper I, Fig. 6 B) and nodules (paper I, Fig. 2 C) speaks against the notion that ird1 expression in blood cell compartments is needed for Toll signaling itself. Both ird1 null and Tl10b larvae showed an abundance of lamellocytes in lymph gland and in circulation (paper I, Fig. 5 B and C). However, only the loss of ird1 expression led to an almost complete down regulation of plasmatocyte in favor of lamellocyte markers. By driving ird1-RNAi expression in different tissues, I could show that ird1 expression in hemocytes but not fat body was required to prevent this commitment of plasmatocytes to the lamellocyte lineage (paper I, Fig. 4 B). Finally, my finding that ird1 null larvae are completely devoid of crystal cells (paper I, Fig. 5 D and Fig. S4 C) can be seen as a final prove that ird1 expression plays an important role in hematopoiesis.
ird1 null larvae lack crystal cells and show melanization defects

Recently it has been shown that of the three phenoloxidases (PO) encoded by the Drosophila genome two are exclusively produced in crystal cells whereas the third may be found also in lamellocytes and cell free hemolymph (Binggeli et al., 2014; Irving et al., 2005b; Nam et al., 2008). My finding that ird1 null larvae lack crystal cells while showing plenty of lamellocytes may therefore explain the seemingly contradicting melanization defects observed by me and others in ird1 null larvae (paper I, Fig. S3 A, B and Wu et al., 2007; Wu and Anderson, 1998). The fact that these animals do not form melanin at the side of a septic injury may indicate that crystal cells are required for this early step of an immune response. In contrast, the high frequency of nodules observed in ird1 null larvae might indicate that their lamellocytes aberrantly clot together and maybe sometimes melanize. Besides the lack of crystal cells, a possible clotting of lamellocytes could also explain why larvae expressing ird1-RNAi in their hemocytes are compromised in their ability to melanize parasitic wasp eggs (paper I, Fig. 5 E). The observation that filter paper incubated together with the injured ird1 null larvae do not stain black (paper I, Fig. S3 B and Wu et al., 2007; Wu and Anderson, 1998), indicates that their hemolymph lacks any residual phenoloxidase activity and possibly that lamellocytes are retained from leaving the body through the wound.

Silencing ird1 in hemocytes increases total number of blood cells in Tl^{cob} mutant larvae

To verify the notion that blood cells lacking ird1 expression are less motile I quantified sessile hemocytes numbers in Tl^{cob} larvae carrying the plasmatocyte reporter eaterDsRed (Sorrentino et al., 2007) and expressing ird1-RNAi in their hemocytes. In agreement with my model, compared to control larvae without the RNAi construct these larvae had on average more blood cells in their sessile compartments (paper I, Fig. 6 A and Fig. S5 A). In addition, loss of ird1 expression in hemocytes also caused an dramatic increase in circulating blood cell numbers found in Tl^{cob} animals (paper I, Fig. 6 B). This rise in total cell number is too big to be accounted for only by the slight increase in blood cell mitosis previously reported to occur in Tl^{cob} larvae (Qiu et al., 1998). Loss of ird1 expression in hemocytes may therefore further increase blood cell mitosis and in addition hinder them from going into circulation. Together this could explain the overall increase in cell numbers I
found in \( Tl^{10b} \) larvae expressing \( ird1 \)-RNAi in their hemocytes. This is supported by our finding that \( ird1 \) null larvae show dramatically increased but usually intact lymph glands, despite the fact that they are filled with lamellocytes (paper I, Fig. 4 B). The increase in number of circulating blood cells in \( Tl^{10b} \) larvae was recently shown by us to be dependent on Tl-signaling in the fat body (paper II). In contrast, expressing \( ird1 \)-RNAi in the fat body did not change the total number of plasmatocytes nor increase nodule frequencies in \( Tl^{10b} \) larvae (paper I, Fig. 6 A, B and suppl. Fig. 5 C).

The implication ensuing from this, that the Tl signaling pathway and \( ird1 \) may function in distinct tissues, was supported by the finding that co-expression of \( ird1 \)-RNAi and UAS-\( Tl^{10b} \) in hemocytes did not lead to an significant increase in total plasmatocyte numbers (paper I, Fig. 6 A and B).

**Silencing \( ird1 \) in hemocytes causes defects in vesicular trafficking**

Previously it has been shown that starvation-induced autophagy is impaired in the fat bodies of \( ird1 \) null larvae causing the accumulation of protein aggregates (Lindmo et al., 2008). Even under normal conditions, I found that blood cells expressing \( ird1 \)-RNAi contained on average significantly more autolysosomes staining with pH sensitive dye Lysotracker red (paper I, Fig. 7 D and G). Consequently the strength of this signal throughout each cell was on average significantly higher compared to wt controls (paper I, Fig. S6 B, to the right). This is in contrast to a previous study that failed to see discrete staining with this dye in fat bodies dissected from starved \( ird1 \) null larvae (Lindmo et al., 2008). This may indicate that the efficiency of the \( ird1 \)-RNAi constructs used is insufficient to completely block the formation of acidic organelles. Our result however is in line with findings made by others that hemocytes extracted from larvae expressing \( vps34 \)-RNAi or being completely devoid of this gene also show more diffused Lysotracker red staining (Velichkova et al., 2010). Using the autolysosome specific \( Atg8 \)-mCherry fusion construct (Nezis et al., 2010; Rahman et al., 2012) we could show that autolysosomes in \( ird1 \)-RNAi expressing cells were on average also significantly smaller compared to controls (paper I, Fig. 7 A and B). Although starvation also increases the size of these vesicles in \( ird1 \)-RNAi expressing cells, they never reached the dimensions of those formed in control hemocytes. Together this indicates that although autophagy is not completely blocked, silencing \( ird1 \) expression in hemocytes causes an aberrant accumulation of autolysosomes and an inability to response to nutritional cues. These findings are consistent with the known role that the \( Vps34 \)-
Vps15 protein complex plays in controlling autophagosome-lysosome fusion in the fat body (Juhász et al., 2008; Lindmo et al., 2008).

When bound to autophagosomes the \textit{Atg8a} fusion constructs emits green light (GFP) (Nezis et al., 2010; Rahman et al., 2012). I found that this signal was more dispersed and on average much stronger in hemocytes expressing \textit{ird1}-RNAi independent of dietary conditions (\textbf{paper I}, Fig. 7 A and suppl. Fig. 6 A to the left). Also in contrast to control cells, starvation did not cause an obvious shift in the localization of the GFP signal towards individual foci. Besides supporting the notion that \textit{ird1} is required in hemocytes to response to dietary stress, the results obtained with the GFP signal from the \textit{Atg8a} fusion construct indicate that also earlier vesicles of the autophagosomal pathway accumulate in blood cells when \textit{ird1} is lacking.

Besides controlling the formation of autophagosomes the \textit{Ird1-Vps34} protein complex also plays an essential role in endosomal trafficking (Meléndez and Neufeld, 2008; Vanhaesebroeck et al., 2010). With no specific antibodies available for \textit{Ird1}, I relied on a GFP tagged FYVE zinc-finger domain containing protein to detect phosphatidylinositol-3-phosphate (PI3P), the product of the \textit{Ird1-Vps34} kinase complex. This lipid is a crucial membrane component of early endosomes, multivesicular bodies and autophagosomes (Christoforidis et al., 1999). The binding of GFP-Myc-2xFYVE to PI3P is limited to its membrane-integrated form (Misra and Hurley, 1999) in early endosomes and multivesicular bodies (Wucherpfennig et al., 2003; Gillooly et al., 2000). As previously seen with the \textit{Atg8-GFP} reporter, expressing \textit{ird1}-RNAi in hemocytes led to in general a much more distorted but stronger GFP-Myc-2xFYVE signal compared to control cells (\textbf{paper I}, Fig. 7 D and suppl. Fig. 6 B to the left). Similarly, it was previously found that the regular localization of GFP-Myc-2xFYVE could be distorted when over expressing \textit{mtm} in hemocytes thereby counteracting Vps34 kinase activity (Velichkova et al., 2010). The Myc-2xFYVE distribution pattern reported in the same study for hemocytes expressing a Vps34 kinase-dead construct bears even more resemblance to our findings. Like the Lysotracker red staining obtained in these cells, silencing \textit{ird1} expression led to GFP-Myc-2xFYVE positive vesicles that were significantly smaller but more frequent than in controls (\textbf{paper I}, Fig. 7 D-F). Together these results indicate that like with autophagosomes and lysosomes, loss of \textit{ird1} expression in hemocytes leads to and aberrant accumulation of early endosomes and/or multivesicular bodies. It has been known from other tissues of the fly such as ovarian follicle cells and the fat body that mutations in endosomal pathway components can affect autophagosomal vesicles as well (Juhász et al., 2008; Rusten et al., 2007). My
results indicate that in hemocyte ird1 acts as a central coordinator for the interconnection of different vesicle trafficking events.

**Tl signaling and loss of ird1 expression causes similar Tl receptor localizations**

Based on a study that found Toll antibody to bind exclusively to the plasma membrane (Hashimoto et al., 1991), it was believed for a long time to be the only place from which the receptor signals. In contrast using the GAL4/UAS system to express a Tl-GFP fusion protein, a more recent study showed that in embryos Toll also localizes in early endosomes marked by the mCherry-Rab5 reporter (Lund et al., 2010). The same study showed that endocytosis of the Toll receptor is crucial for the generation of the Dorsal gradient in the embryo and can be artificially induced by expressing a UAS-Tl\textsuperscript{10b}-GFP construct. As our previous assays indicated that ird1 loss-of-function and Tl gain-of-function often lead to similar phenotypes, I wanted to determine if this is also the case for the localization of the Toll receptor.

Compared to hemocytes fat body cells are considerably bigger making it easier to image a possible translocation of the Toll receptor. Therefore, I first expressed Tl-GFP or Tl\textsuperscript{10b}-GFP with or without ird1-RNAi in fat body cells marked by the mCherry-Rab5 fusion construct. In the embryo Tl-GFP localization was shown to oscillate between being membrane bound and more vesicular depending on the exact embryonic stage (Lund et al., 2010). In fat bodies dissected from different wild type larvae, I found Tl-GFP expressing sometimes forming a grind-like pattern on the cell surface but most often localized below the membrane in small vesicles (paper I, Fig. 8 A). The reason for this difference may be that the Tl signaling pathway was activated by dissection in some larvae, causing a translocation of the receptor. Allthough larvae used for the experiments were meticulous staged it cannot be excluded that some of them may have differed in age. The fat body is known to disintegrates during metamorphosis. The concentration of Tl-GFP in small vesicles beneath the membrane may be an early sign of this process. However, the observed effect in fat bodies from wild-type was never as strong as the seen in organs dissected from larvae expressing Tl\textsuperscript{10b}-GFP. Co-expression of ird1-RNAi with Tl-GFP could phenocopy this effect of a constitutively activated Toll pathway. Although in both situations the size and number of GFP positive vesicles dramatically increased compared to controls, I never saw much overlap with early endosomes marked by the mCherry-Rab5 reporter. Similar results were obtained by expressing these constructs
specifically in hemocytes (paper I, Fig. 8 D). However as seen before in this study, when the HmlΔGAL4 driver was used there was a lot of variation from cell to cell even within the same blood sample. This is again likely due to the fact that silencing ird1 expression forces plasmatocytes into the lamellocyte linage.

It has been previously noted that the exact mechanisms of Tl signaling can vary between development and immunity as shown by the different roles that Dif and dorsal play (Manfruelli et al., 1999; Meng et al., 1999; Rutschmann et al., 2000). This may explain the fact that in contrast to embryos (Lund et al., 2010), Tl-GFP and mCherry-Rab5 positive structures rarely overlapped in fat bodies or blood cells that were analyzed in this study. This may indicate that the localization of Tl to early endosomes is much shorter during an immune response. Alternatively, the translocation of the receptor into the cytoplasm may take an entirely different route in larval compared to embryonic tissues. Either of these scenarios may also explain why GFP positive vesicles in fat bodies dissected from UAS-TlΔ 10b or ird1-RNAi expressing larvae penetrated much deeper into the cytoplasm compared to controls, sometimes even uto the level of the nucleus. Vesicles so far from the cell membrane are usually not bound by Rab5 any longer (Vanhaesebroeck et al., 2010). It would therefore be interesting to test in the future if Tl localizes predominantly in late endosomes in response to immune stimuli. Nevertheless, the fact that the expression of either TlΔ 10b-GFP or ird1-RNAi could lead to a dramatic increase in size and number of mCherry-Rab5 positive vesicles indicates that early events of endosomal trafficking may still play a central role in Tl pathway activation even in immunity.

Silencing ird1 in hemocytes causes morphological defects

The results presented so far suggest indirectly that loss of ird1 causes blood to forfeit their ability to react to immune stimuli by going into circulation. Previously it has been shown that hemocytes expressing RNAi constructs targeting expression of central autophagy regulator gene Atg1 or Vps34 binding factor myotubularin (myo), are not properly recruited to wound sites artificially inflicted upon Drosophila larvae (Kadandale et al., 2010; Velichkova et al., 2010). Besides showing similar autophagy defects as reported here for blood cells lacking ird1 expression, hemocytes expressing Atg1 or myo-RNAi also showed morphological defects. To check for similar phenotypes blood cells were extracted from control larvae, ird1 homo and heterozygotes and animals expressing ird1-RNAi with Cg-Gal4. After the blood cells were allowed to spread on glass slides they were fixated and stained with a antibody
(α-tubulin) and a toxin (Phalloidin) that are coupled to fluochromes in order to visualize the actin and microtubules of the cytoskeleton. Like reported for cells expressing Atg1 or myo-RNAi (Kadandale et al., 2010; Velichkova et al., 2010), blood cells with a reduced ird1 gene dosage showed clear spreading defects. Starting as round cells when placed on the glass slides, they did not over time flatten out or extend many spiky protrusions as observed for control cells (paper I, Fig. 9 A). As a consequence, they had a much-reduced overall cell size mostly due to the fact that they failed to form the F-actin rich apron usually surrounding the cell (quantified in paper I, Fig. 9 B).

In line with the role of ird1 in autophagy, similar defects have previously been observed by others when hemocytes were treated with class III PI3K inhibitor 3-methyladenine (Kadandale et al., 2010; Wu et al., 2010). Driving RNAi constructs targeting expression of other central regulators of the autophagy pathway such as Atg4, Atg6, Atg7, Atg8a, and Atg9 also led to similar defects (Kadandale et al., 2010). The same study showed that the obtained phenotypes were not due an indirect effect of depriving cells of energy but that autophagy plays an important role in the remodeling of blood cell architecture possibly by interfering with the Rhô1 pathway. In stark contrast to results obtained by us with ird1 null and ird1-RNAi expressing larvae, animals lacking the Atg1 gene had wt blood cell numbers in circulation and sessile compartment (Kadandale et al., 2010). Similar results were obtained when expressing mtm-RNAi specifically in hemocytes (Velichkova et al., 2010). This indicates that although ird1 plays a similar role as mtm or Atg1 in regulating autophagy and blood cell morphology it may have an additional function in maintain blood cell homeostasis possibly through suppressing mitosis. Our hypothesis that ird1 may be needed for blood cell motility gets further support from the finding that mtm-RNAi expressing cells form clumps in the sessile compartment (Velichkova et al., 2010). As this may be the reason that they cannot be recruited to wound sides (Velichkova et al., 2010), similarly hemocytes lacking ird1 expression may be incapable of going into circulation in response to Toll signaling as shown by me.

The notion that among the genes regulating vesicle transport systems ird1 has a prescient position is supported by our finding that loss of ird1 expression can force plasmatocytes into the lamellocyte linage (paper I, Fig. 9 A next to arrow, Fig. 4 A, B and Fig. 5 A-C). Studies on Atg1, mtm or its pseudophosphatase Su(var)3-9 and 'Enhancer of zeste' (SET, (Tschiersch et al., 1994)) domain binding factor Sbf, did not report lamellocytes forming when these genes were knocked out in the Drosophila larva (Kadandale et al., 2010; Velichkova et al., 2010; Jean et al., 2012).
However on glass slides covered with lecting Concanavalin A, Atg1-RNAi expressing blood cells do exhibited a discoid shape that resembles some of the lamellocytes we extracted from ird1 null larvae ((Kadandale et al., 2010) and paper I, Fig. 9 A). Similarly, RNAi mediated knock down of mtm resulted in hemocytes having a larger footprint on a glass slide and displaying smooth or ruffled edges devoid of protrusions also resembling lamellocyte morphology (Velichkova et al., 2010). It is therefore possible that if blood cell type specific antibodies would have been used in these studies, some of these cells could have been classified as lamellocytes.

Further literature reflection

When analyzing mutant null larvae for another autophagy related protein known to bind to the Vps34-Ird1 kinase complex called Atg6 (Beclin-1 in mammals) (Funderburk et al., 2010), Eric Baehrecke and co-workers found that the lymph glands of these animals readily stain with the L1 antibody (Shravage et al., 2013). I found similar lamellocyte specific staining in lymph glands of ird1 null larvae (paper I, Fig. 5 C). Also the down regulation of plasmatocyte specific markers in these organs is a common feature in Atg6 and ird1 null larvae. Like ird1, loss of Atg6 also affected the pattern formation and expression of plasmatocyte markers by blood cells in the sessile compartment. Maybe most importantly, both ird1 and Atg1 null larvae frequently show nodules and die as late third instars or early pupae (Wu et al., 2001; Wu et al., 2007; Shravage et al., 2013).

As regulators of the autophagy pathways, mutants both ird1 or Atg6 mutants show vesicle trafficking defects. As shown by us in ird1-RNAi expressing hemocytes, mitotic clonal fat body cells deficient for Atg6 failed to properly form mCherry-Atg8a positive vesicles in response to starvation (Shravage et al., 2013). In the same study it was shown that in contrast to their wild-type neighbours, fat body cells lacking Atg6 do not localize a GFP fused FYVE domain containing protein to punctate structures. In fact, mutant fat body cells did not show any GFP signal above background levels. This is in stark contrast to our finding that expressing ird1-RNAi in hemocytes causes a variable but significant increase in the relative strength of the GFP signal coming from the FYVE containing protein (paper I, suppl. Fig. 6 C). There are several possible explanations for this apparent discrepancy. The trivial reason may be that cells of different origin were analyzed, or that the gene loss-of-function situation was brought about in different ways. Alternatively and likely more interesting is the possibility that Atg6 is more important for the activity of the Vps34 kinase, whereas Ird1 helps ensuring the proper localization of its product. This may explain why the
distribution of the GFP-FYVE reporter signal was diffuse in ird1-RNAi expressing cells sometimes even covering the nucleus (paper I, Fig. 7 D).

Also when it comes to the localization of Rab5 positive early endosomes my observations differ from results obtained by expressing Atg6-RNAi in the fat body. While silencing ird1 caused an increase in early endosomes especially in the perinuclear space (paper 8, Fig. 7 A), expression of Atg6-RNAi was reported to have the exact opposite effect (Shravage et al., 2013). The fact that I used confocal microscopy while the study on Atg6 relied on regular fluorescence microscopy may however partly explain these differences.

In contrast to the sometimes-contradictive results obtained when monitoring vesicular trafficking events, when it comes to blood cell phenotypes similarities outweigh the differences. Both ird1 and Atg6 null larvae have melanotic nodules in contrast to animals lacking Atg7, Atg13 and Atg8 mutants (Shravage et al., 2013). Similarly like in ird1/- animals, these nodules may be the result of an increased circulating blood cell number that differs ten fold between Atg6 null and control larvae (Shravage et al., 2013). In contrast to wild type genetic background, blood cell phenotypes obtained in this study when expressing ird1-RNAi in hemocytes of Tl<sup>ob</sup> larvae were much more severe (paper I, Fig. 6 A and B). Likewise expression of a full length UAS-Atg6 construct with Hml<sup>Δ</sup>GAL4 could not suppress nodule formation in Atg6<sup>-/-</sup> larvae (Shravage et al., 2013). This indicates that although loss of either Atg6 or ird1 expression is sufficient to block autophagy cell autonomous for a strong effect on blood cell phenotypes cues from another tissue are required. Shravage et al. (2013) tested the possibility that the Toll or Imd signaling pathways are involved in bringing about nodule formation in Atg6 null mutants. However, double mutants being null for Atg6 and lacking Dif, dorsal or Relish still showed strong nodulations phenotypes. In contrast, we collected strong evidence that ird1 loss-of-function phenotypes can be attenuated by constitutive Toll signalling from the fat body. This may indicate that although Toll signaling is not required for the establishment of hemocyte defects in autophagy mutants, its activation may affect their severity.
PAPERS II + III

Results/Discussion

Mutants rendering the important immune signaling pathway Toll constitutively active such as Tl\textsuperscript{10b} have been known to show hematopoietic defects since a long time (Lanot et al., 2001; Qiu et al., 1998). Even though they have been used frequently as models to investigate the activation of the cellular immune system in flies and mammals (Dearolf, 1998; Evans et al., 2003), the tissue chiefly responsible for emitting the immune stimulus in these mutants was still unknown.

In accordance to previous studies, we found that the overall number of circulating blood cells is increased in Tl\textsuperscript{10b} mutant larvae. Also the animals showed lamellocytes and nodules, which are usually absent in bodies of wt larvae (paper II, Fig. 4 A, B and Fig. 3 A). Both of these phenotypes are mainly brought about by plasmatocytes, which constituting the vast majority of blood cells and give rise to lamellocytes (Honti et al., 2014; Honti et al., 2010). Accordingly, our lab could show in a previous study that expressing an UAS-Tl\textsuperscript{10b} construct in hemocytes with the hml\textsuperscript{Δ}-GAL4 driver is sufficient to mimic some of the blood cell activation phenotypes known to occur in Tl gain-of-function mutants or in fly larvae infected by a parasitic wasp (Zettervall et al., 2004). An obvious assumption was therefore that the hemocyte phenotypes in Toll\textsuperscript{10b} mutants are caused by Toll signaling in the blood cells themselves.

Our partner lab in Tampere, Finland had conducted a genome wide RNAi screen in Drosophila S2 cells (paper III). They monitored Drosomycin promoter driven luciferase activity in cell cultures whose immune response had been triggered by either having a constitutively active Toll receptor (Tl\textsuperscript{10b}) or by heat killed E. coli bacteria. Silencing expression of important components of both Toll and Imd pathways in these cells like Myd88 or Relish was shown to potently decrease luciferase activity (paper III, Fig. 1 A). Also when being transfected with dsRNA coding for 23 additional genes of the Drosophila genome, Drosomycin luciferase activity could be reproducibly decreased (paper III, Fig. 1 B). Among the genes previously not known to affect NF-κB signaling in the fly was one coding for the G protein-coupled receptor kinase 2 (Gprk2). The sequence of this gene is highly conserved in evolution. Like in zebrafish and some mammals, where it is known as GRK5, it contains a regulator of G protein signaling domain (paper III). Like in irdt (paper I) its sequence also codes for a serine/threonine kinase and it belongs to a
protein family known to control the activity of GTPase proteins. Another candidate gene that was discovered by our Tl<sup>lob</sup> deletion screen, Rab23, codes for a member of this protein family. As decreasing the gene dosage of either irdi or Rab23 was shown by me to modify blood cells phenotypes of Tl<sup>lob</sup> mutants (paper I), similar results were expected for Gprk2.

For this, fly stocks carrying RNAi constructs targeting Gprk2 and 10 other genes identified as candidates in the cell culture screen were crossed individually to hml<sup>ΔGFP</sup>; Tl<sup>lob</sup> flies. As described in paper I, the offspring of these crosses was graded according to the appearance of their sessile hemocyte-banding pattern. As positive controls, I included fly strains harboring Myd88, Dif, or dl-RNAi constructs. In contrast to expectations however none of the RNAi construct tested with our assay was able to reproducibly suppress the loss-of-sessile hemocyte phenotype when expressed in blood cells of Tl<sup>lob</sup> larvae (data not shown). The first possible interpretation for this was that some of the candidates may have been false positives, or that the RNAi constructs used were not efficient enough to significantly silence endogenous expression of these genes. However, our partner lab in Finland could subsequently show that when being expressed in the fat body, some of these RNAi lines including those targeting Gprk2 and Myd88 could reduce Drosomycin mRNA production in response to a septic injury (paper III, Fig. 3 A-C). Besides verifying that these genes also regulate Toll signaling in vivo, this strongly suggested that the failure to pick up an measurable effect with our assay was not due lack of RNAi efficiency.

From this we hypothesized that if expressing Myd88-RNAi was sufficient to block the TI pathway in blood cells but this did not affect the loss of sessile cells in Tl<sup>lob</sup> larvae, than the most important signaling in this mutant must come from yet another tissue. To evaluate the impact that TI signaling from various parts of the larval body has on the activation of the cellular immune system, we expressed UAS-Tl<sup>lob</sup> with a set of GAL4 drivers having different tissue specificities. As published by our lab before (Zettervall et al., 2004), driving UAS-Tl<sup>lob</sup> expression specifically in hemocytes was sufficient to significantly increase the number of circulating hemocytes (plasmatocytes and lamellocytes) and to disrupt the sessile hemocyte-banding pattern (paper II, Fig 1 A, B and D and E). These effects of could be suppressed by co-expressing UAS-Tl<sup>lob</sup> with either Gprk2 or Myd88-RNAi constructs with Hml<sup>ΔGAL4</sup> (paper II, Fig. 1 A, B and D and E; paper III, Fig. 7 C and data not shown). Besides verifying the efficiency of the Myd88-RNAi construct with our assay,
this showed that like in the fat body Gprk2 functions as a regulator of Tl signaling in the hemocytes.

Compared to the effect of an activated Tl signaling in the hemocytes, the impact on blood cell number was however much stronger with some of the other tested drivers. The total number of circulating plasmatocytes and lamellocytes increases more than ten-fold when I used the fat body specific FB-Gal4 driver to express UAS-Tl\textsuperscript{10b} suggesting a proliferative response (paper II, Fig. 1 D and E). By using the plasmatocytes specific reporter eater-GFP (Kroeger et al., 2011) we could also show that larvae expressing UAS-Tl\textsuperscript{10b} in their fat body had a distorted sessile hemocytes banding pattern (paper II, Fig. 1 C). Compared to hemocyte specific expression, driving UAS-Tl\textsuperscript{10b} in both blood cells and fat body (with Cg-GAL4 (Asha et al., 2003)) or in the midgut (with NP\textsuperscript{3084}-Gal4) also gave a stronger increase in circulating blood cell numbers (paper II, Fig. 1 D and E). As with Hml\textsuperscript{Δ}GAL4, co-expression UAS-Tl\textsuperscript{10b} with Myd88-RNAi could partially suppress these effects. From these experiments we concluded that although Tl signaling from the hemocytes is sufficient to induce blood cell phenotypes, other tissues like the fat body seem to play a more important role.

To test if this was also the case in the genetic background of the Tl\textsuperscript{10b} mutant allele we again took advantage of fluorescent plasmatocyte markers (Hml\textsuperscript{Δ}GFP and/or eater-GFP) to visualize sessile blood cell populations inside living third-instar larvae. As expected from our results obtained with UAS-Tl\textsuperscript{10b} construct, the presence of the Tl\textsuperscript{10b} allele caused a disruption of the sessile hemocyte-bandning pattern in both Hml\textsuperscript{Δ}GFP and FB-GAL4 genetic backgrounds (paper II, Fig. 2 B and E). By crossing the Myd88-RNAi fly line to these stocks, I could show that only by blocking Toll signaling in the fat body not in the hemocytes was it possible to suppressed the loss of sessile bands phenotype in Tl\textsuperscript{10b} larvae (paper II, Fig. 2 C, F and quantified in G). Accordingly, while circulating plasmatocyte numbers in Tl\textsuperscript{10b} larvae were unaffected by expressing Myd88-RNAi with Hml\textsuperscript{Δ}GFP, driving the construct with FB-GAL4 reduced blood cell concentrations to an almost wild-type level (paper II, Fig. 4 A). By using MSNF\textsuperscript{9mo-mCherry} (hereafter called msn-Cherry) reporter (Tokusumi et al., 2009) we could also show for the first time that besides lamellocyte in circulation (Lanot et al., 2001; Qiu et al., 1998), Tl\textsuperscript{10b} mutant and UAS-Tl\textsuperscript{10b} expressing larvae display this cell type in their sessile compartments (paper II, Fig. 1 B’, C’ and Fig. 3 G and I). The lamellocyte often formed clots under the integument that were not obviously attached to internal organs, although the fat body of Tl\textsuperscript{10b} larvae sometimes showed signs of desintegration (paper II, suppl Fig. 1 C and data not shown). Apart
from expressing the *msn-Cherry* reporter many of these cell accumulation showed melanization making them visible as nodules in the larval body (*paper II*, Fig. 3 B, D and G and I). In accordance with our model, blocking Tl signaling by silencing *Myd88* expression in the fat body was sufficient to suppress these phenotypes (*paper II*, Fig. 3 E, J and K and I). Unexpectedly however, expressing *Myd88*-RNAi in hemocytes of *Tl*10b larvae enhanced the number of lamellocytes in the sessile compartments and percentage of larval offspring showing nodules (*paper II*, Fig. 3 C, H and K). In summary, these experiments demonstrate for the first time that apart from producing and secreting antimicrobial peptides for the humoral response (Hultmark, 2003) the fat body also plays a central role in controlling cellular immunity. Although dispensable for the development of blood cell defects, Tl signaling in hemocytes may however in a negative feedback loop be required for attenuating their severity.

**Further literature reflection**

The loss of sessile band phenotype went unnoticed in previous studies that used the *Tl*10b allele (Lanot et al., 2001; Qiu et al., 1998). This is likely due to the fact that no blood cell specific reporters were available at the time. In contrast, I was probably the first to notice the lymph gland malformation in *Tl*10b larvae (*paper I, paper II*, Fig. 2 B and E) for a very different reason. When introducing the *Hml*ΔGFP and FB-GAL4 into the *Tl*10b fly line, we may have changed its genetic composition. It is possible that we have unintentionally out-crossed mutants suppressing the Tl signaling pathway that have accumulated in the genetic background of this stock over the years. This would also explain why in our hands crosses to the GAL4 containing *Tl*10b stocks regularly resulted in more than 50% of the larval offspring showing at least one nodule (*paper II*, Fig. 3 A-L). In contrast, previous studies using the original *Tl*10b stock reported frequencies between 1-5% (Lanot et al., 2001; Qiu et al., 1998).

With the help of plasmatocyte specific markers we could analyze the morphological features of *Tl*10b lymph glands (and where blood cell reporters are expressed) in more detail. As described previously for the plasmatocyte marker *eater* coupled to *dsRed* (Sorrentino et al., 2007), lymph glands dissected from wt control larvae showed expression throughout the entire length of the organ (*paper II*, Fig. 1 I). Larvae that are heterozygote for *Tl*10b are known to show lamellocytes in circulation (Lanot et al., 2001; Qiu et al., 1998), which likely derive from differentiated plasmatocytes (Honti et al., 2014; Honti et al., 2010; Márkus et al., 2010).
However in the lymph glands dissected from $T^{lo}$ mutant larvae, *eaterDsred* was still expressed in all parts of the organ (paper II, Fig. 1 J). This is in line with other results obtained by me showing that this reporter is still visible in hematopoietic tissue even when the cellular immune system is activated (paper I).

In the same study, I could also show that a substantial proportion of circulating blood cells extracted from $T^{lo}$ animals show a decreased $Hm\tilde{l}^\Delta GFP$ signal. This was likely due to the fact that as plasmocytes differentiate into lamellocytes they turn down production of Hemolectin, thereby shutting down GAL4 transcription. However, in $T^{lo}$ mutant lymph gland lobes not only was $Hm\tilde{l}^\Delta GFP$ still visible but its expression was no longer restricted to parts of the most anterior lobes as in the wild-type control (paper II, Fig. 2 I-J’’). As $Hm\tilde{l}^\Delta GFP$ is a marker for differentiated plasmocytes (Sinenko and Mathey-Prevot, 2004), this indicates that in situations of a constitutive Toll signaling pro-hemocytes (that usually occupy the medullary zone and the secondary lobes of the lymph gland (Crozatier and Meister, 2007) may differentiate prematurely. This in turn may cause the primary lymph gland to burst prematurely explaining why it cannot be found in the vast majority of $T^{lo}$ larvae. Further, together with the primary lymph gland lobes the posterior signaling center may be lost as well. This small pocket of cells is usually positioned between primary and secondary lobes and thought to hinder pro-hemocytes of the medullary zone from going into differentiation (Krzemien et al., 2007). The posterior signaling center exerts this control amongst others by secreting Hedgehog signaling molecules that activate the respective pathway in the neighboring cells of the medullary zone (Mandal et al., 2007). It is conceivable that the posterior signaling center can also control the cells of the secondary lobes in a similar fashion. Its loss may therefore induce differentiation of all cells irrespective of their position in the lymph gland. This may explain the $Hm\tilde{l}^\Delta GFP$ expression pattern observed by us in lymph glands dissected from $T^{lo}$ larvae (paper I, Fig. 3 C and paper II, Fig. 2 J-J’’’) and why lamellocytes can be found throughout these organs (paper I, Fig. 5 C). An alternative explanation for the morphological differences seen between lymph gland from wild-type and $T^{lo}$ larvae could be that in situations of constitutive Toll signaling this organ is never properly formed. Indeed, when investigating lymph glands of first and second instar larvae carrying the $T^{lo}$ allele, I noticed that their lobes were generally smaller compared to same aged wild type larvae (data not shown). Also, as observed in many lymph glands dissected from third instar $T^{lo}$ larvae (paper I, Fig. 5 C and paper II, Fig. 2 J-J’’’), there was no major difference in size between the most anterior and more posterior pairs of lobes. Without a reporter visualizing the posterior
signaling center it was therefore impossible to decide if the primary lymph gland lobes in these animals never properly form or burst prematurely during development.

Our partner lab in Tampere could verify that Gprk2 also plays a role in T1 signaling in a more natural context, by infecting flies expressing Gprk2-RNAi in their fat bodies with the pathogen B. bassiana. Compared to control animals without the RNAi construct, adult flies with silenced Gprk2 expression showed reduced mRNA levels of the Drosomycin gene (paper III, Fig. 6 A-C). In addition they could show that these flies also died faster than control flies after a septic injury with M. luteus (paper III, Fig. 7 A-B). In our lab we could show that larvae expressing irdi-RNAi specifically in their hemocytes were reduced in their ability to melanize parasitic wasp eggs (paper I, Fig. 5 E). In contrast, blocking T1 signaling by expressing Myd88-RNAi in fat body had no apparent effects on the immune response against the parasite. Wasp eggs dissected from larvae at different time points after infection neither showed more melanization nor did the killing of the parasite affected much (paper II, Fig. 5 A and B). This may indicate that in contrast to T10b mutant larvae, Toll signaling in the fat body of wild type larvae does not play a major role in cellular immunity. Alternatively, the wasp may inject compounds together with the egg that actively suppress Toll signaling thereby obscuring our assay. Indeed we could find evidence that the ability of larvae to activate Drosomycin-GFP reporter in their fat bodies is affected by the stage at which its immune system is in fighting off the parasite (paper II, Fig. 5 C and D). It is therefore likely that by conducting additional immune assays in the future we could answer the question what importance T1 signalling in the fat body has for the cellular immune response of Drosophila in the a more natural context. This will likely also help us to better understand the basic mechanisms of inter-organ communication during innate immune responses in humans and other mammals.
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