Thomas Hauling

# The Activation of the Insect Immune System by Endogenous Danger Signals with Emphasis on *Drosophila melanogaster*

kassel university press

Thomas Hauling

# The Activation of the Insect Immune System by Endogenous Danger Signals with Emphasis on *Drosophila melanogaster*

kassel university press

This work has been accepted by the faculty of Mathematics and Natural Sciences of the University of Kassel as a thesis for acquiring the academic degree of Doktor der Naturwissenschaften (Dr. rer. nat.).

Supervisor: Prof. Dr. Wolfgang Nellen, Universität Kassel
Co-Supervisor: Prof. Dr. Ulrich Theopold, Stockhoms universitet

Defense day: 29th August 2011

Bibliographic information published by Deutsche Nationalbibliothek
The Deutsche Nationalbibliothek lists this publication in the Deutsche Nationalbibliografie; detailed bibliographic data is available in the Internet at http://dnb.d-nb.de.

Zugl.: Kassel, Univ., Diss. 2011
ISBN print: 978-3-86219-230-4
ISBN online: 978-3-86219-231-1
URN: http://nbn-resolving.de/urn:nbn:de:0002-32319



Printed in Germany

**Teile meiner Dissertation sind als Publikation in einer Fachzeitschrift veröffentlicht worden:**

Activation of insect phenoloxidase after injury: endogenous versus foreign elicitors. Bidla G, Hauling T, Dushay MS, Theopold U. J Innate Immun. 2009 Jun;1(4):301-8. Epub 2008 Oct 30.

Ich habe das Protokoll zur Peptidoglykaninjektion in *Galleria* und *Drosophila* Larven etabliert (Abschnitte 3.9 und 4.1.2). Der *In vitro* Assay zur Messung von PPO-Aktivierung (Abschnitt 3.8) ist von Gawa Bilda optimiert worden und von ihm und mir durchgeführt worden. *Dot blot* und *Hanging drop* Präparationen sowie mikroskopische Studien sind von Gawa Bidla und mir durchgeführt worden (Abschnitte 3.6, 3.7 und 3.8.1). Ich habe das TARGET System (Abschnitt 3.17) etabliert, welches unter anderem zur Induktion von Melanisierung in Larven *in vivo* verwendet worden ist.
Sämtliche Experimente zur Untersuchung von PPO-Aktivierung durch aberranten Zelltod (ab Seite 64) sowie das gesamte Projekt *The activation of the innate immune system by altered self in Drosophila larvae* (ab Seite 65) sind von mir durchgeführt worden.

**Nicht enthalten in meiner Dissertation sind Ergebnisse aus folgenden Publikationen:**

Evidence for an immune function of lepidopteran silk proteins. Korayem AM, Hauling T, Lesch C, Fabbri M, Lindgren M, Loseva O, Schmidt O, Dushay MS, Theopold U. Biochem Biophys Res Commun. 2007 Jan 12;352(2):317-22. Epub 2006 Nov 14.

Clotting factors and eicosanoids protect against nematode infections. Hyrsl P, Dobes P, Wang Z, Hauling T, Wilhelmsson C, Theopold U. J Innate Immun. 2011;3(1):65-70. Epub 2010 Oct 16.

## Zusammenfassung auf deutsch

Wie das Immunsystem auf fremde „Nichtselbst"-Signale und auf Gefahrensignale (*danger signals*), ausgelöst durch „verändertes Selbst" (*altered self*), reagiert ist nur unvollständig verstanden. Gefahrensignale entstehen zum Beispiel durch aberrantes Gewebswachstum, damit verbundenen zellulären Stress und Zelltod. Wie und ob solche Ereignisse das Immunsystem in Insekten aktiveren können war Thema meiner Arbeit. Die Melanisierungsreaktion mit Phenoloxidase (PO) als Kernenzym ist eines der Hauptimmunmechanismen in Insekten. Melanisierung ist einfach mit dem Auge erkennbar und photometrisch messbar und eignet sich daher sehr gut als Indikator für die Aktivierung des Immunsystems. PO hat Ähnlichketen mit Tyrosinasen in Vertebraten und katalysiert die Oxidierung von Mono- und Diphenolen zu Orthoquinonen, welche nichtenzymatisch zu Melanin polymerisieren. Einige der reaktiven Intermediate habe zytotoxische Aktivität und wirken vermutlich als Zytokine. Die Aktivierung von PO ist daher streng reguliert. Dazu trägt bei, dass PO als inaktives Zymogen (PPO) in spezialisierten Immunzellen, den Kristallzellen (*crystal cells*) gespeichert und an die Hämolymphe abgegeben wird, wo es enzymatisch gespalten und so aktiviert wird. Zusammen mit Gawa Bidla habe ich untersucht wie PPO in der Hämolymphe und während der Hämolymphgerinnung (*clotting*) in Insekten aktiviert wird. In zwei Dipteren, der Fruchtfliege *Drosophila melanogaster* und in der Mücke *Anopheles gambiae* fand Melanisierung lokal begrenzt im Hämolymphkoagulum (*clot*) statt. Die Melanisierungsmuster waren anders in der Wachsmotte *Galleria mellonella* und im Schmetterling *Pararge aegeria* (deutscher Name: Waldbrettspiel). Statt lokal begrenzt, trat Melanisierung in den beiden Lepitopteren systemisch in der Hämolymphe auf. Grundsätzlich können Immunreaktionen sowohl durch exogene, fremde Elizitoren als auch durch endogene Gefahrensignale ausgelöst werden. In *Galleria* und *Drosophila* untersuchte ich, welche Faktoren zur PO-Aktivierung beitragen. In *Galleria* wurde in einem *in vitro* Assay Melanisierung durch die bakteriellen Zellwandbestandteile Lipopolysaccharid (LPS) und Peptidoglykan (PGN) induziert. Im Gegensatz zur Melanisierung in *Galleria* und zur systemischen Aktivierung von PO nach Sepsis war PO-Aktivierung in *Drosophila* Hämolymphe nicht empfindlich gegenüber bakteriellen Elizitoren. Stattdessen

aktivierte endogenes Phosphatidylserin PO *in vitro*. Phosphatidylserin ist ein Phospholipid, das sich in gesunden Zellen auf der Plasmamembraninnenseite befindet, jedoch unter anderem bei Apoptose auf der Membranaussenseite exponiert wird und so für das Immunsystem erkennbar wird. Entsprechend führte die ektopische Induktion von Apoptose in Larven zu Melanisierung *in vivo.* Endogene Elizitoren hatten in *Galleria* keinen signifikanten Einfluss auf PO-Aktivität. Dass Phospholipide und in bestimmtem physiologischen Kontext Apoptose eine Immunantwort in *Drosophila* induzieren können, hat mich veranlasst die Interaktion zwischen Immunsystem und verändertem Selbst näher zu untersuchen. Mittels RNAi knockdown von Tumorsuppressorgenen oder Exprimierung einer dominant-aktivem Form des Protoonkogens Ras ($Ras^{V12}$) habe ich tumorähnliches Gewebswachstum in *Drosophila* Larven induziert. Flügelimaginalscheiben (die Voräuferorgane der adulten Flügel), in denen das Tumorsuppressorgen *lethal (2) giant larvae* reprimiert wurde oder $Ras^{V12}$ exprimiert wurde, wuchsen aberrant und melanisierten während der Metamorphose im Puppenstadium. Speicheldrüsen, in denen $Ras^{V12}$ exprimiert wurde, zeigten ebenfalls übermässiges Wachstum gegenüber dem Wildtyp und produzierten wie maligne Tumore in Vertebraten Matrixmetalloprotease (MMP). Spaltprodukte aufgrund von MMP Aktivität sind interessanterweise als Gefahrensignale in Insekten identifiziert worden. $Ras^{V12}$ exprimierende Speicheldrüsen (und Fettkörper) wurden von Hämozyten infiltriert und zum Teil eingekapselt. Die Einkapselungsreaktion in Insekten weist signifikante Ähnlichkeit mit Granulomabildung in Vertebraten auf. Einkapselungen waren in einigen Fällen melanisiert. An der Einkapselung waren alle drei Hämozyttypen beteiligt, die in *Drosophila* Larven vorkommen: Plasmatozyten, Lamellozyten und Kristallzellen. Diese zelluläre Reaktion war von humoraler Expression des mutmaßlichen Opsonins Tep1 begleitet, welches auch in Einkapselungsreaktionen gegen Eier parasitärer Wespen hochreguliert wird. Genetische Mutanten mit defekten Kristallzellen und vermutlich beeinträchtigter Phagozytose ($Bc^{1}$) zeigten verstärkte Einkapselung aber melanisierten nicht. Einkapselung / Melanisierung und abberantes Gewebswachstum der Speicheldrüsen waren verstärkt in Eiger (*Drosophila* Tumornekrosefaktor) Mutanten. $Ras^{V12}$ Speicheldrüsen wiesen Merkmale von Nekrose und Apoptose in den Bereichen auf, wo Hämozyten anhafteten. Meine Ergebnisse zeigen, dass aberrantes Gewebe und damit verbundene Gefahrensignale, wie extrazelluläres

Phosphatidylserin und vermutlich weitere Zellstress-/ Zelltodsignale komplexe Immunantworten in *Drosophila* auslösen können. Wie in Vertebraten spielen auch in *Drosophila* infiltrierende Immunzellen und Tumornekrosefaktor eine Rolle bei der Eindämmung von aberrantem, tumorähnlichem Gewebe. *Drosophila* könnte als Modell zur Erforschung von Gefahrensignalen als Auslöser von Autoimmunreaktionen (beispielsweise gegen Tumore) dienen.

## Abstract

How the immune system reacts to foreign, non-self and endogenous so-called danger signals is incompletely understood. Danger signals emerge i.e. as a consequence of aberrant overgrowth of tissue, which in turn is often associated with cellular stress and cell death. Tissue that exposes danger signals to the immune system is often referred to as altered self to discriminate it from self, which by definition does not elicit an immune response. The aim of my dissertation was to investigate how altered self can activate the immune system in insects.

The melanization reaction with phenoloxidase (PO) as its core enzyme is one of the key immune mechanism in insects. Melanization is visible by eye and can be measured photometrically. It hence provides a simple readout for immune activation. Similar to vertebrate tyrosinases, PO catalyzes the oxidation of mono and diphenols to orthoquinones, which polymerize non-enzymatically to melanin. Several of the reactive intermediates have cytotoxic activity. Hence, PO activity has to be tightly controlled. In parts this is achieved by storing the enzyme in specialized blood cells (crystal cells) as an inactive zymogen – prophenoloxidase (PPO). I tested how PPO is activated in the hemolymph and during clotting in insects. Melanization was locally restricted in the clot of two Dipterans, *Drosophila melanogaster* and *Anopheles gambiae*. In contrast, the patterns of melanization were different in two Lepidopterans, *Galleria mellonella* and *Pararge aegeria*. Instead of being locally restricted melanization occurred systemically throughout the hemolymph. In general, immune reaction can be activated by exogenous, foreign elicitors as well as by endogenous danger signals. In *Galleria* and *Drosophila* I studied which factors contribute to PPO activation. In an *in vitro* assay melanization was superinduced by bacterial cell wall components Lipopolysaccharid (LPS) and Peptidoglycan (PGN). Unlike systemic PO activation in *Galleria*, PO activation was not sensitive to bacterial elicitors in the *in vitro* assay. Instead, the inner-membrane phospholipid phosphatidylserine (PS) superinduced PO-activity. While hidden from immune recognition in healthy cells, PS translocates to the extracellular site of the plasma membrane during apoptosis. In line with this observation, endogenous stimuli as a consequence of ectopic apoptosis triggered melanization *in vivo*.

In *Galleria*, endogenous elicitors did not have a significant impact on PO activity.

Having observed that phospholipids and apoptosis under certain physiological conditions could activate the immune system in *Drosophila* I continued to investigate the interactions between the innate immune system and altered self.

Via knockdown of tumor suppressor genes and by expressing a dominant active form of the proto-oncogene Ras ($Ras^{V12}$) I induced tumor-like aberrant growth in larval tissue and characterized the immune response.

Repression of the tumor suppressor *lethal (2) giant larvae* or expression of $Ras^{V12}$ in wing imaginal discs (the precursor organs of the adult wings) led to aberrant overgrowth and melanization of adult wings.

Similarly, $Ras^{V12}$ expression in salivary glands resulted in overgrowth and production of matrixmetalloprotease (MMP). MMP is produced by many malignant tumors and facilitates metastasis by catalyzing the breakdown of tumor surrounding extracellular matrix (ECM). Interestingly, ECM fragments have been shown to stimulate immune responses in insects.

$Ras^{V12}$ expressing salivary glands (and fat body) were infiltrated by *Drosophila* blood cells (hemocytes) and partially encapsulated. Encapsulation shares significant similarites with granuloma formation in vertebrates. Encapsulated tissue melanized in some cases. All three hemocyte classes that are observed in *Drosophila* larvae were involved in the encapsulation process: plasmatocytes, lamellocytes and crystal cells. Cellular immunity was accompanied by humoral expression of the putative opsonin Tep1, which is also upregulated during the cellular response against parasitoid eggs. Both hemocyte infiltration of aberrant salivary glands and tissue overgrowth were increased in a genetic mutant with non-functional crystal cells und a putative defect in phagocytosis. Encapsulated glands never melanized indicating that crystal cell borne PO is essential for the process. Overgrowth and hemocyte infiltration with encapsulation as the ulimate consequence were also enhanced in mutants with compromised Eiger (*Drosophila* tumor necrosis factor) function. $Ras^{V12}$ salivary glands stained positive for indicators of necrosis and apoptosis and cell death correlated with adhering hemocytes.

My data show that aberrant tissue and associated danger signals, such as extracellular PS and putatively additional signals related to cellular stress and cell death can trigger complex immune responses in *Drosophila*. Like in vertebrates,

infiltrating immune cells and tumor necrosis factor are involved in control of tumor-like, aberrant tissue. Hence, *Drosophila* might serve as a model to study danger signals as inducers of autoimmune responses against altered self (i.e. tumors).

## List of abbreviations

| | |
|---|---|
| AMP | Antimicrobial peptide |
| approx | approximately |
| aTEP1 | *Anopheles* Thiolester containing protein 1 |
| Bc | Black cells |
| Bf | Bright field |
| Bl | Bleed |
| BM | Basement membrane |
| BSA | Bovine serum albumin |
| Bx | Beadex |
| CD36 | cluster of differentiation 36 |
| CED-1 | Cell death abnormality 1 |
| CGEQ | Cysteine, Glycine, Glutamic acid, Glutamine |
| CNS | Central nervous system |
| $CO_2$ | Carbondioxide |
| Da | Daughterless |
| DABCO | 1,4-diazabicyclo[2.2.2]octane |
| DAMP | Danger associated molecular pattern |
| DAP-type | Diaminopimelic acid type |
| DAPI | 4',6-diamidino-2-phenylindole |
| dIAP1 | *Drosophila* inhibitor of apoptosis 1 |
| dIAP2 | *Drosophila* inhibitor of apoptosis protein 2 |
| Dif | Dorsal-related immunity factor |
| dl1 | Discs large 1 |
| DNA | Deoxyribonucleic acid |
| DOPA | 3,4-dihydroxyphenylalanine |
| DREDD | Death-related ced-3/Nedd2-like protein |
| Dscam | Down syndrome cell adhesion molecule |
| ECM | extracellular matrix |
| EDTA | Ethylenediaminetetraacetic acid |
| Egr | Eiger |
| FA | Formaldehyde |
| FITC | Fluorescein isothiocyanate |
| FLICA | Fluorescent Labeled Inhibitor of Caspases |
| GAP | Guanosine-triphosphatase-activating protein |
| GDP | Guanosine diphosphate |
| GFP | Green fluorescent protein |
| GNBP | Gram negative bacteria binding protein |
| GTP | Guanosine-triphosphatase |
| GTPase | Guanosine-triphosphatase |
| Hid | Head involution defective |
| IBM | IAP-binding motif |
| IKK | Inhibitor of nuclear factor kappa B kinase |
| IMD | Immune deficiency |
| JAK/STAT | Janus Kinase/Signal Transducer and Activator of Transcription |
| JNK | c-Jun N-terminal kinase |

| | |
|---|---|
| l(2)gl | Lethal (2) giant larvae |
| LOF | Loss of function |
| LPS | Lipopolysaccharide |
| Lys-type | Lysine-type |
| M1/2 | Macrophage 1/2 |
| MAMP | Microbe associated pattern |
| MAPK | Mitogen activated protein kinase |
| MAPKKK | Mitogen-activated kinase kinase kinase |
| Mcr | Macroglobulin complement-related |
| MMP | Matrix Metalloproteinase |
| MP | Melanization Protease |
| MP1 | Melanization Protease 1 |
| MP2 | Melanization Protease 2 |
| Nedd | Neural precursor cell expressed |
| NF-kappaB | Nuclear factor kappa B |
| NK cell | Natural killer cell |
| OD | Optical density |
| Oe | Oenocyte |
| PAS | Phenoloxidase activating system |
| PAE | PPO activating enzyme |
| PBS | Phosophate buffered saline |
| Pc | Phase contrast |
| PC | Phosphatidylcholine |
| PGN | Peptidoglycan |
| PGRP | Peptidoglycan recognition protein |
| PGRP-LC | Peptidoglycan recognition protein LC |
| PGRP-LE | Peptidoglycan recognition protein LE |
| proI | Propidium iodide |
| PI | Phosphatidylinositol |
| Ppl | Pumpless |
| PO | Phenoloxidase |
| proPO | Prophenoloxidase |
| PS | Phosphatidylserine |
| PSR | Phosphatidylserine receptor |
| PTEN | Phosphatase and tensin homolog |
| $Rab5^{DN}$ | Rab-protein 5 $^{dominant\text{-}negative}$ |
| Rac1 | Ras-related C3 botulinum toxin substrate 1 |
| Rac2 | Ras-related C3 botulinum toxin substrate 2 |
| RHD | Rel homology domain |
| RIP1 | Receptor interacting protein 1 |
| RNA | Ribonucleic acid |
| RNAi | Ribonucleic acid interference |
| RNS | Reactive nitrogen species |
| ROS | Reactive oxygen species |
| RT | Room temperature |
| S2 | Schneider 2 |
| SC-Cl | Scavenger receptor Cl |
| Scrib | Scribbled |
| Sgs3 | Salivary gland secretion 3 |

| | |
|---|---|
| siRNA | Small interfering ribonucleic acid |
| SP7 | Serine protease 7 |
| SPE | Spätzle processing enzyme |
| Spn27A | Serpin 27A |
| Spn28D | Serpin 28D |
| TAB2 | TAK1-binding protein 2 |
| TAK1 | TGF-beta activated kinase 1 |
| TAM | Tumor-associated macrophage |
| TARGET | Temporal and regional gene expression targeting |
| TCT | Tracheal cytotoxin |
| TEP | Thioester containing protein |
| TGF-beta | Transforming growth factor beta |
| TIMP | Tissue inhibitor of metalloproteases |
| Tl | Toll |
| TLR | Toll-like receptor |
| TNF | Tumor necrosis factor |
| TNFR | Tumor necrosis factor receptor |
| TRITC | Tetramethylrhodamine isothiocyanate |
| tubGAL80$^{ts}$ | Tubulin GAL80 $^{temperature\text{-}sensitive}$ |
| UAS | Upstream Activation Sequence |
| Upd 1 - 6 | Unpaired 1 – 6 |
| VDRC | Vienna *Drosophila* RNAi Center |
| wt | Wild type |

## Table of Contents

## Aim of this work

The activation of the innate immune system by endogenous factors as opposed to foreign molecular patterns, i.e. of bacteria or fungi is poorly understood.

Such immune-stimulatory endogenous factors (or danger signals) are assumed to emerge as a consequence of cell stress and necrotic cell death.

In insects, phenoloxidase-catalyzed melanization provides an easy read-out for immune activation. Phenoloxidase (PO) is often considered the key enzyme of arthropod immunity, relevant for the defense against microbial infections, parasites and important for efficient wound healing. Although it is conceptionally understood that PO is released as an inactive zymogen termed prophenoloxidase (PPO), which is activated by a cascade of serine proteases, it is debated how this cascade is initiated, i.e. the initial stimuli that lead to PPO activation are poorly understood. Studies suggest that despite being present throughout the phylum arthropoda, the mode of PPO activation differs between species (Cerenius, Lee et al. 2008).

I intended to shed light on the importance of putative danger signals supposedly present on altered self compared to known microbial elicitors as activators of the innate immune system in insects. Danger signals can also be exposed or released by tumors; e.g. many vertebrate tumors elicit immune responses, apparently due to the presence of specific danger signals that are absent in normal tissues. I addressed the specific contribution of the innate immune system during control of tumor growth in a follow-up study. The aim was to genetically induce tumor-like overgrowth specifically in non-immune organs and to investigate the immune response against such aberrant tissues. *Drosophila* was chosen as a model system for this study since powerful genetic tools are available for this organism, which can be utilized to induce aberrant self and to manipulate the immune response.

# 1 Introduction

According to classical self non-self models of immune activation, foreign elicitors derived from non-self, e.g. microbe-associated molecular patterns (MAMPs) are recognized by immune receptors of the host followed by immune activation. However, more recently studies suggest that the immune system can also be triggered by endogenous molecules in addition to foreign elicitors as originally proposed by Matzinger (Matzinger 2002). Host-derived intracellular molecules that are released or exposed upon injury or stress are thought to act as danger signals, which are recognized by the immune system. Hence the term "danger model" has been coined as opposed to classical self-nonself models of immune activation (Matzinger 2002). In analogy to MAMPs, danger signals are also referred to as danger-associated molecular patterns (DAMPs). Cells or tissue that exposes or releases DAMPs is referred to as altered-self to distinguish it from self, which is non-immunogenic by definition.

In vertebrates, mutations in genes that have a role in the response to danger signals or that are involved in clearing dead cells can lead to autoimmune reactions (Deane and Bolland 2006; Nagata 2007; von Landenberg and Bauer 2007; Ehrchen, Sunderkotter et al. 2009).

In *Drosophila*, numerous autoimmune mutants have been described. The mutants contain melanotic nodules in their hemolymph, most of which are assumed to originate from autoimmune reactions to apparently altered-self (Watson, Johnson et al. 1991; Nappi, Vass et al. 2004; Minakhina and Steward 2006). Melanization is carried out by phenoloxidase (PO), which in turn is stored as an inactive zymogen prophenoloxidase (PPO) in specialized patrolling immune cells (hemocytes), termed crystal cells. Melanotic mutants have been classified dependant on the nature of the melanized tissue. So called class 1 melanotic mutants are defined by an intact hematopoietic system reacting against aberrant non-immune tissue (Watson, Johnson et al. 1991). It has been shown that in some of these mutants tissues are infiltrated by hemocytes, which form layers (encapsulation – see paragraph 1.2.5) on the tissue (Minakhina and Steward 2006). Once encapsulated, PPO is activated, ultimately resulting in capsule melanization. In addition to such melanotic pseudo-tumors, the

formation of neoplastic tumors has been demonstrated in *Drosophila*, which fulfill most criteria of vertebrate tumors, i.e. continued proliferation, escape from cell death, loss of differentiation and metastasis (Brumby and Richardson 2003). Also, aberrant overgrowth has been induced in larval salivary glands, which escaped developmental degradation during pupal stage (Berry and Baehrecke 2007). While an involvement of cellular immunity has been described against melanotic mutants and more recently against invasive tumors originating from imaginal tissue, immune responses against aberrant salivary glands have not been reported. In general the interactions between the immune system and aberrant self have not been characterized in detail and are just beginning to emerge.

To provide a better understanding of my work I will give a brief introduction to insect immunity in general and *Drosophila* immunity in particular. Furthermore, I will discuss concepts in immunity and tumor modeling in *Drosophila*, followed by a short overview over the technical advantages of using *Drosophila melanogaster* as a model system.

## 1.1 A brief introduction to the immune system of insects

Unlike vertebrates, insects rely solely on innate defense mechanisms. Vertebrate-like adaptive immunity, which is mediated by lymphoid lineage derived blood cells such as B-cells and T-cells is absent in insects (Lavine and Strand 2002). Instead insects have developed a powerful innate immune system consisting of humoral and cellular defense mechanisms, which enables them to mount specific immune responses against different immune insults. Humoral factors that are secreted into the hemolymph include anti-microbial peptides (AMPs), reactive oxygen species (ROS), Reactive Nitrogen Species (RNS), proteolytic cascades that lead to hemolymph clotting and melanization (Boman and Steiner 1981; Steiner, Hultmark et al. 1981; Nappi, Vass et al. 1995; Bulet, Hetru et al. 1999; Nappi, Vass et al. 2000; Theopold, Li et al. 2002; Molina-Cruz, DeJong et al. 2008). Cellular defense mechanisms include phagocytosis, nodulation and encapsulation (Lavine and Strand 2002). Generally, insect immunity is mostly mediated by the fat body, an organ analogous to the vertebrate liver and via patrolling leukocyte-like immune cells, termed hemocytes.

Under certain conditions, the expression of immune effectors can also be observed in the gut, trachea, testis and the salivary glands (Ferrandon, Jung et al. 1998; Ha, Oh et al. 2005; Ha, Oh et al. 2005; Senger, Harris et al. 2006).

Once pathogens have breached physical barriers such as the larval cuticle, the adult exoskeleton, the peritrophic membrane of the mid-gut or the chitinous lining of the trachea they face an effective clotting system, which sequesters intruders to a forming matrix and which can even specifically target microbes (Wang, Wilhelmsson et al. 2010). Microbes that escape clotting may be phagocytosed. Objects that are too large to be engulfed such as eggs injected into the larval hemocoel by parasitoid wasps are often encapsulated (Russo, Dupas et al. 1996; Lavine and Strand 2002). Immune insults usually elicit transcription and secretion of humoral effector molecules such as AMPs and opsonins, the latter supporting phagocytosis. Most AMPs are specifically effective against certain types of microbes, e.g. in *Drosophila*: Drosomycin is active against fungi, Diptericin against Gram-negative bacteria and Defensin targets Gram-positive bacteria (Meister, Lemaitre et al. 1997; Imler and Bulet 2005; Lemaitre and Hoffmann 2007).

The production organs for humoral factors are the fat body, epithelial tissues and hemocytes. AMPs and other humoral factors required for clotting and melanization are mostly synthesized in the fat body. The development of hemocytes (hematopoiesis) occurs during embryogenesis and later during larval development in the lymph gland. Mature hemocytes mostly develop from precursor cells, termed prohemocytes. At least in *Drosophila*, differentiation into mature hemocytes might also occur outside of the lymph gland and from already differentiated hemocytes (Markus, Laurinyecz et al. 2009; Stofanko, Kwon et al. 2010). Granulocytes represent the most abundant hemocyte class in *Lepidopterans* and *Anophelidae*. They function as professional phagocytes, like plasmatocytes in *Drosophila*. Confusingly, Lepidopteran plasmatocytes appear to be most similar to *Drosophila* lamellocytes in terms of morphology and function (Ribeiro and Brehelin 2006). Both cell types participate in encapsulation. Specialized hemocytes termed crystal cells in *Drosophila* and oenocytoids in other insects including *Anophelidae* and *Lepidopteran* species store and release the PO zymogen PPO. At least in the mosquito *Anopheles gambiae*

PPO expression can also be induced in granulocytes upon an immune challenge (Castillo, Robertson et al. 2006).

## 1.2 The *Drosophila* immune system

*Drosophila* and vertebrates share many key factors of immune signaling pathways including Toll/ Toll-like Receptor (TLR), Immune deficiency (IMD)/ Tumor Necrosis Factor Receptor (TNFR), Janus Kinase/ Signal Transducer and Activator of Transcription (JAK/STAT) and c-Jun N-terminal kinase (JNK) as well as effector molecules such as antimicrobial peptides (AMPs) that are involved in the innate immune response (Lemaitre and Hoffmann 2007).

*Drosophila* hematopoiesis gives rise to three different lineages of hemocytes: plasmatocytes, crystal cells and lamellocytes. Plasmatocytes share similarities with mammalian blood cells of the monocyte/ macrophage lineage (Williams 2007). In general, higher vertebrates use a broader repertoire of specialized innate immune cells compared to insects. Plasmatocytes are the most abundant cell type in flies, constituting approx. 95% of all hemocytes in larvae. Crystal cells represent about 5% of the larval hemocyte population. They contain crystal-like inclusions (hence their name) of PPO, the key enzyme for immune-defense related melanization and wound healing. Lamellocytes encapsulate objects too large to be phagocytosed such as eggs from parasitoid wasps and aberrant tissue (Meister and Lagueux 2003; Minakhina and Steward 2006). Although rarely found in healthy larvae lamellocytes can occur in significant numbers in certain genetic backgrounds. Lamellocytes have not been observed in adult flies. Crystal cells and lamellocytes do not have clear homologues in vertebrates but signaling pathways that control their differentiation have in parts mammalian counterparts (Meister 2004).

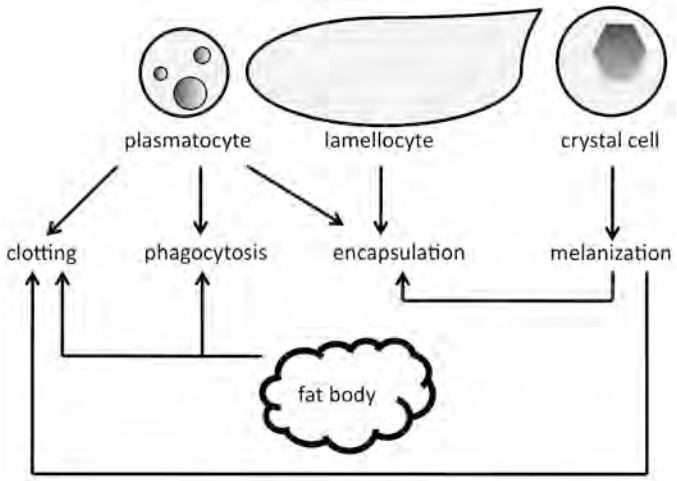


**Figure 1. Overview of the larval immune defense in *Drosophila*.** Three types of mature hemocytes can be distinguished in larvae the majority of which patrol the hemolymph. Another fraction of hemocytes is sessile and found mostly underneath the larval cuticle. Plasmatocytes participate in phagocytosis of apoptotic cells and microbes, encapsulation of larger objects and hemolymph clotting that seals wounds and entraps microbes. Lamellocytes appear to be exclusively involved in encapsulation. Crystal cells secrete prophenoloxidase, which is the key enzyme of the melanization cascade. Melanization in turn is involved in clotting and required for complete encapsulation. The fat body represents the main organ of the humoral defense. It secretes immune factors functioning in hemolymph clotting and phagocytosis and also secretes antimicrobial peptides that are directly involved in pathogen killing.

### 1.2.1 Signaling pathways that regulate immune gene expression

The insect immune system, which is best understood in *Drosophila* and hence used here as an example, is tightly regulated by signaling pathways that are partially conserved in innate immunity of higher vertebrates. The two most investigated pathways are the Toll and the Immune Deficiency (IMD) pathway (De Gregorio, Spellman et al. 2002). It has been estimated that they control the expression of about 80% of all immunity related genes. JAK/STAT signaling has been implicated in antiviral immunity, encapsulation of parasitoid eggs and has been shown to be important in regulation of hemocyte proliferation in response to tissue damage and tumor development in larvae (Dostert, Jouanguy et al. 2005; Williams, Wiklund et al. 2006; Pastor-Pareja, Wu et al. 2008). The JNK pathway has a role in general hemocyte activation, encapsulation and control of AMP expression (Delaney, Stoven et al. 2006; Williams, Wiklund et al. 2006; Bidla, Dushay et al. 2007). The immune signaling pathways in *Drosophila* fat body are illustrated in Figure 2.

#### *1.2.1.1 The Toll pathway*

The Toll pathway plays an essential role in establishing the dorso-ventral axis of *Drosophila* embryos as well as in several other developmental programs. The fly genome encodes of family of 9 Toll receptors with only Toll (Tl, CG5490) itself clearly linked to immune defense. The other eight receptors show expression patterns in embryogenesis and metamorphosis suggesting developmental roles (Ferrandon, Imler et al. 2007). In immunity, Toll receptors mediate expression of those AMPs, which are mostly effective against fungi and Gram-positive bacteria, Drosomycin being the most prominent one. Mammalian Toll-like receptors function as pattern-recognition receptors, i.e. they are directly activated by foreign ligands (e.g. bacterial lipopolysaccharide (LPS)) or DAMPs originating from altered self such as mitochondrial DNA and heat-shock proteins (Tsan and Gao 2004; Zhang, Raoof et al. 2010). The activation of *Drosophila* Toll is different: In flies, Toll is triggered via the cleaved form of the cytokine Spätzle, which in turn is proteolytically activated by Spätzle Processing Enzyme (SPE). SPE itself is activated via proteolytic cascades that in turn are triggered by microbe-specific recognition receptors PGRPs (peptidoglycan recognition protein), GNBPs (Gram-negative binding protein) and by endogenous mechanisms involving the serine protease Persephone. PGRPs recognize the bacterial cell wall component peptidoglycan (PGN). In the case of Spätzle-activating PGRPs this is lysine (lys)-type PGN, which is mostly found in Gram-positive bacteria. GNBPs detect bacteria but also seem to play a role in the recognition of fungi (Valanne, Wang et al. 2011).

Upon binding of activated dimerized Spätzle to Toll, the receptor in turn forms a homodimer in the plasma membrane. This causes recruitment of cytoplasmic factors Pelle, dMy88 and Tube ultimately resulting in the release of two REL transcription factors Dorsal and Dif, which then translocate from the cytoplasm into the nucleus where they ultimately activate transcription of numerous effector genes, including the AMP Drosomycin, which is nearly exclusively controlled by Toll signaling and hence commonly used as a read-out gene for the activity of this pathway.

#### *1.2.1.2 The IMD pathway*

Two PGRPs are associated with Immune Deficiency (IMD) signaling, PGRP-LE and PGRP-LC (which has three isoforms). All PGRP-LCs are transmembrane receptors whereas PRGP-LE is assumed to be an intracellular receptor as it lacks a transmembrane domain but might also be secreted into the hemolymph to act extracellulary(Kurata 2010). Common to both PGRPs is that they form oligomers upon binding of diaminopimelic acid (DAP)-type PGN or shorter PGN fragments, e.g. tracheal cytotoxin (TCT). Once clustered, the receptors signal via N-terminal domains. IMD putatively functions immediately downstream of PGRP-LC, which it binds to, although binding itself might not be crucial for IMD activation (Aggarwal and Silverman 2008). IMD is a death domain protein, similar to RIP1, a key molecule of the mammalian TNFR signaling pathway. Downstream of IMD, signal transduction is mediated via the MAPKKK transforming growth-factor beta activated kinase 1 (TAK1), which is required for activation of the IKK (IκB kinase) complex (Vidal, Khush et al. 2001). The molecular mechanism by which IMD and TAK1 interact remains to be elucidated. It has been suggested that the *Drosophila* Inhibitor-of-Apoptosis2 (dIAP2) and the apical caspase DREDD (Death related ced-3/Nedd2-like protein) have a role in this process (Kleino, Valanne et al. 2005). TAK1 likely functions in complex with TAK1-binding protein 2 (TAB2), leading to simultaneous induction of two downstream branches of the pathway that eventually result in NF-kappaB/Relish and JNK activation (Zhuang, Sun et al. 2006). Relish activation is dependent on the IKK complex and on DREDD, both of which are required for proteolytic activation of a transcription factor module from Relish, RHD. Once cleaved off, RHD translocates from the cytoplasm into the nucleus where it activates transcription of the AMP Diptericin and other immune effectors. It has been proposed that Ras-MAP kinase signaling inhibits IMD signaling but not Toll signaling in larvae and adult flies in the absence of immune insults (Ragab, Buechling et al. 2011).

#### *1.2.1.3 The JNK pathway*

The role of JNK signaling in immunity has not been fully understood. Several studies indicate that the pathway is involved in the inhibition of AMP expression (Kim, Yoon et al. 2005; Kim, Choi et al. 2007). However, there is also evidence supporting a contributing role of JNK signaling in IMD-mediated AMP upregulation (Kallio, Leinonen et al. 2005; Delaney, Stoven et al. 2006).

Generally, JNK is induced by stress stimuli (e.g. osmotic stress, irradiation, and oxidative stress). The pathway is activated upon wounding and required for wound healing processes (developmental processes) such as embryonic dorsal closure, thoracic closure, and for closure of adult epithelial wounds and damaged imaginal discs (Ramet, Lanot et al. 2002; Bosch, Serras et al. 2005). It has been shown that TAK1 activates JNK signaling in response to bacterial infection via the JNK kinase basket (Boutros, Agaisse et al. 2002). At least in hemocyte-derived S2 cells activation of JNK induces cytoskeleton remodeling (Sawamoto, Winge et al. 1999). It has hence been suggested that JNK has a role in activation of hemocytes (Boutros, Agaisse et al. 2002; Bidla, Dushay et al. 2007).
It has also been shown that the *Drosophila* homologues of Tumor Necrosis Factor (TNF), Eiger and Tumor Necrosis Factor Receptor (TNFR), Wengen activate apoptosis via JNK. This process has been shown to be relevant for crystal cell activation (Bidla, Dushay et al. 2007) and also recently for elimination of epithelial tumor cells (Igaki, Pastor-Pareja et al. 2009).

#### *1.2.1.4 The JAK/STAT pathway*

Another pathway that plays a role in embryogenesis is the JAK/STAT pathway with its three main components domeless receptor, Janus kinase JAK and STAT transcription factor. In immunity JAK/STAT signaling controls the expression of Turandot stress factor proteins (of unknown function) and of thioester-containing proteins, TEPs, which share sequence homology with vertebrate complement factor C3 and are believed to act as opsonins; i.e. they aid phagocytosis by binding to matter that is targeted for engulfment (Stroschein-Stevenson, Foley et al. 2006). JAK/STAT signaling can be activated by cytokines of the unpaired (Upd1 – Upd3)

family that bind to the receptor Domeless. All three Unpaired proteins appear to be induced upon wounding, gut infections and tumorigenesis in larvae (Agaisse, Petersen et al. 2003; Pastor-Pareja, Wu et al. 2008; Jiang, Patel et al. 2009). In all three situations this leads to increased hemocyte proliferation via a self-amplifying feed back loop, i.e. Upds are secreted at the wound site, by the gut or tumor leading to expression and secretion of Upd3 in hemocytes that in turn induces production of these cytokines in the fat body. At least in tumors and wounds JNK signaling has been suggested to trigger *upd* expression (Pastor-Pareja, Wu et al. 2008).

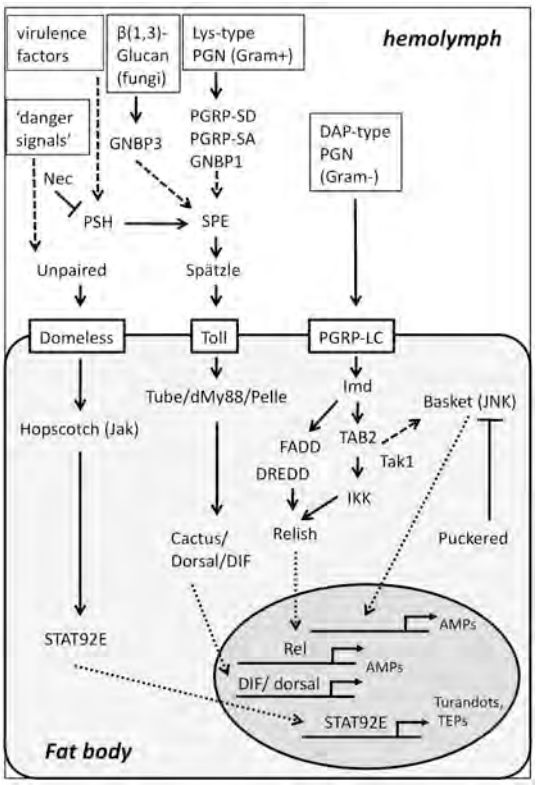


**Figure 2. Immune Signaling pathways in the larval fat body.** Jak/STAT pathway: Endogenous danger signals that originate from wounding and tumours lead to secretion of cytokines of the unpaired family that in turn bind Domeless receptor. Domeless activates the Janus kinase Hopscotch, which autophosphorylates. STAT92E binds to Hopscotch and translocates to the nucleus after phosphorylation, where it activates transcription of stress response associated Turandots and Thioester-binding proteins (TEPs) that putatively act as opsonins. Toll pathway: Fungal virulence factors trigger Toll signaling via Persephone (PSH), which can be inhibited by the serpin Necrotic (Nec). PSH activates Spätzle-activating enzyme (SPE), resulting in cleavage of Spätzle to its mature form, which dimerizes and binds dimerized Toll. This leads to the recruitment of Tube dMy88 and Pelle. By an unknown mechanism, Cactus is phosphorylated causing its degradation by the proteasome. As a consequence DIF and Dorsal translocate to the nucleus where they activate transcription of immune genes including antimicrobial peptides (AMPs). The Imd pathway: DAP-type PGN is recognized and bound by membrane-bound PGRP-LC (PGRP-LE has been omitted for simplicity). Upon PGN binding, PGRP-LC recruits Imd. Imd interacts with FADD, which in turn binds the caspase DREDD. DREDD is believed to cleave Relish once Relish is phosphorylated. Cleavage splits Relish into an inhibitory domain that remains in the cytoplasm and the Rel domain, which migrates into the nucleus to activate transcription of AMPs (and other immune genes). Phosphorylation is carried out by the IKK complex, which itself is putatively activated by TAB2 and Tak1 in a Imd-dependent manner. Tak1 can trigger JNK signaling, which in turn can contribute to induction of AMP expression (There is also evidence for an IMD-inhibitory role of JNK). *Drosophila* JNK, Basket is inhibited by Puckered.

### 1.2.2 *Drosophila* hematopoiesis

Development of *Drosophila* hemocytes occurs in at least two phases, initially during embryogenesis and later during larval stage. Embryonic hemocytes that originate from the procephalic mesoderm are termend plasmatocytes or embryonic macrophages since they phagocytose apoptotic corpses. Crystal cells are derived from

a region near the anterior part of the gut. Their function in embryogenesis is not clear. Differentiation of crystal cells is dependent on the transcription factor Lozenge. The second phase of hematopoiesis occurs in the larval lymph gland and in designated compartments that line the cuticle of 2nd and 3rd instar larvae (Meister and Lagueux 2003; Meister 2004; Markus, Laurinyecz et al. 2009). The lymph gland contains precursor cells that can give rise to all three classes of hemocytes. Triggered by ecdysone at the end of the 3rd larval instar before onset of pupation, prohemocytes differentiate into large numbers of plasmatocytes that continue to exist in adult flies along with plasmatocytes derived from larval embryogenesis. In healthy larvae, hemocytes are released from the lymph gland at the onset of pupation. However, infection and inflammation can result in hemocyte release during the larval stage although this process also appears to be developmentally regulated (i.e. ecdysone-dependent) (Sorrentino, Carton et al. 2002). Lamellocytes are assumed to differentiate in response to specific immune insults and as a consequence of wounding (Markus, Kurucz et al. 2005; Williams 2007). They have been reported to originate both from the lymph gland and from sessile hemocyte populations lining the epidermis (Crozatier and Meister 2007; Stofanko, Kwon et al. 2010). It is generally believed that lamellocytes as well as crystal cells are larval stage specific since they have not been observed in adult flies.

### 1.2.3 Phagocytosis of apoptotic cells and pathogens

In mammals, phagocytosis-competent cells can be divided into professional and non-professional phagocytes, the latter also having other functions in addition to phagocytosis. In *Drosophila*, glial cells can engulf cell debris from the developing central nervous system (CNS) during embryogenesis (Sonnenfeld and Jacobs 1995; Elliott and Ravichandran 2008). Otherwise, phagocytosis is carried out by plasmatocytes, the fly's professional phagocytes. Hemocoel-invading microbes, apoptotic cells and generally particles small enough to be phagocytosed can be engulfed by plasmatocytes (Stuart and Ezekowitz 2008).

The mechanisms by which plasmatocytes recognize and engulf their targets are poorly understood. Uptake of apoptotic cell debris depends on the receptor *croqemort*, which is homologous to the mammalian family of CD36 scavenger

receptors (Franc, Heitzler et al. 1999). In mammals CD36 acts in concert with the PS receptor PSR, of which there is a homologue in the fly. Yet functional evidence for an involvement of this receptor in removal of apoptotic corpses is missing. Another receptor implicated in phagocytosis is *Draper*, the *Drosophila* homologue of *C. elegans* CED-1. It has been suggested that calreticulin serves as an eat-me signal in draper-dependent phagocytic engulfment (Manaka, Kuraishi et al. 2004).

In addition to receptors with apparent specificity for apoptotic cells, *Drosophila* expresses receptors, which recognize bacteria and fungi. A well-characterized one is *Eater* (Erturk-Hasdemir and Silverman 2005; Kocks, Cho et al. 2005). *Eater*-depleted flies show a severe reduction in phagocytosis of both Gram-positive and Gram-negative bacteria. Other receptors involved in phagocytosis of microbes are *Drosophila* Scavenger Receptor Cl (Ramet, Pearson et al. 2001), Down syndrome cell adhesion molecule (Dscam) and Pestes. Dscam was originally discovered as a factor that is essential for CNS development (Schmucker, Clemens et al. 2000). The fact that splicing of *dscam* transcripts can potentially produce about 18.000 isoforms and the observation that Dscam is expressed in plasmatocytes led to the idea that it might have a novel adaptive immunity–type immune function similar to immunoglobulins, which exist in great variety due to somatic recombination and somatic hypermutation (Watson, Puttmann-Holgado et al. 2005). CD36-like Pestes is another receptor that putatively confers specificity to phagocytosis since in S2 cells, only entry of *M. fortuitum* and *L. monocytogenes* is blocked, while the engulfment of *E. coli* and *S. aureus* is not affected (Philips, Rubin et al. 2005).

### 1.2.4 Opsonisation – the putative role of TEPs

Phylogenetically, TEP proteins from insects share sequence similarities with both the vertebrate complement factors C3/C4/C5 and the alpha2-macroglobulin family of protease inhibitors. Complement is a central element of vertebrate innate immunity as it initiates and coordinates defense reactions against microbes and altered self by recognizing changes in surface properties (Ricklin, Hajishengallis et al. 2010). Complement factors bind directly to the microbial or aberrant surfaces thereby initiating their destruction.

TEPs contain a hypervariable region that corresponds to the bait domain of alpha2-macroglobulin and to the anaphylatoxin domain in C3. Most TEPs share a common four-amino-acid (CGEQ) motif defining the thioester-site, to enable formation of covalent bonds with target-surfaces.

In *Drosophila*, the TEP family is composed of six genes (TEP1 - 6), one of which (TEP5) does not seem to be expressed. TEP6 (or Macroglobulin Complement Related, MCR) differs from other TEPs as it contains a serine instead of a cysteine residue in the thioester-binding site thereby putatively rendering the motif non-functional. Most *tep* genes are expressed in hemocytes but can also be induced in the fat body upon septic injury. While the expression of TEPs in *Drosophila* S2 cells enhances phagocytosis of bacteria and fungi by opsonisation, the function of TEPs *in vivo* remains obscure. Flies that are deficient in Tep2, 3 and 4 survive like wildtype controls in infection studies encompassing a panel of bacteria and fungi (Bou Aoun, Hetru et al. 2011).

The function of TEPs has been mostly studied and understood in *Anopheles* where TEP1 (aTEP1) is secreted by hemocytes to act as an opsonin to promote phagocytosis of Gram-positive and Gram-negative bacteria (Blandin and Levashina 2004). Furthermore, aTEP1 has been shown to bind to the surface of *Plasmodium* ookinetes in complex with additional factors thereby facilitating lysis and melanization of the parasite (Blandin, Shiao et al. 2004; Povelones, Waterhouse et al. 2009).

### 1.2.5 Encapsulation

Encapsulation is carried out by plasmatocytes and lamellocytes and occurs as a response against objects that are too large to be phagocytosed and has also been described as a reaction against aberrant self in certain genetic backgrounds. The process is analogous to granuloma formation in vertebrates (Hogan, Weinstock et al. 1999). Encapsulation has mostly been described as a defense mechanism against eggs that are laid by parasitic wasps into the hemocoel of 2$^{nd}$ instar larvae. Egg deposition elicits a massive release of plasmatocytes from the lymph gland and the hemocyte compartments that line the larval epidermis. Once parasitism is recognized, plasmatocytes change from a non-adhering to an adhesive state. They attach to the

egg, spread and form septate junctions, eventually resulting in a primary layer that surrounds the egg. Lamellocytes also strongly proliferate upon wasp parasitism and adhere onto the plasmatocyte layer where they form a second, tight capsule that is often melanized as a consequence of PPO activation. Crystal cells and possibly lamellocytes are the source of PPO. Encapsulated eggs remain in the hemocoel and persist until adult stage.

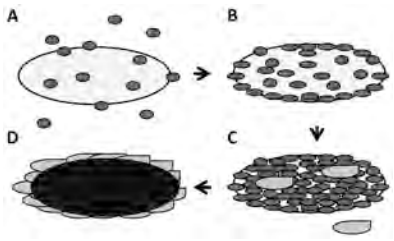


**Figure 3. Schematic cartoon of encapsulation of wasp eggs.** (A) Patrolling plasmatocytes (dark grey) adhere to the egg (white) and (B) spread to ultimately form septate junctions between each other (C). A tight layer of plasmatocytes emerges onto which lamellocytes (light grey) adhere (requiring integrins), which in turn form a capsule around the primary layer (D). PO-dependant melanization (black) can occur as the final step of encapsulation approximately 40h post-parasitization.

Encapsulation of eggs of the parasitoid wasp *Leptopilina boulardi* is severely impaired in Rac1 and Rac2 mutants (Williams, Ando et al. 2005; Williams, Wiklund et al. 2006). Both proteins belong to the family of Rho GTPases, which are essential in regulation of cell migration and cell shape. In Rac2 mutants plasmatocytes and lamellocytes recognize and adhere to the egg but fail to spread and to form septate junctions (Williams, Ando et al. 2005). Why encapsulation is impaired in Rac1 mutants remains to be elucidated. Moreover, *Drosophila* ß$_2$-integrin (Myosperoid) seems to be required for lamellocyte attachment to the egg (Irving, Ubeda et al. 2005). Three immune pathways have been demonstrated to be involved in the encapsulation of parasitoid eggs. Both the Toll and the JAK/STAT pathway are induced upon wasp parasitization and are necessary for a proper encapsulation response. Furthermore, for yet unknown reasons JNK mutants fail to encapsulate wasp eggs (Williams 2007). Another unsolved mystery is how hemocytes actually recognize the egg. However, the disruption of basement membrane has been proposed to trigger encapsulation of (altered) self tissue (Rizki and Rizki 1980).

### 1.2.6 Clotting / coagulation

Similar to vertebrates, *Drosophila* larvae readily form an insoluble clot matrix that stops bleeding and prevents infections upon wounding (Theopold, Schmidt et al. 2004). Factors that are involved in *Drosophila* clotting include van-Willebrand-factor-like Hemolectin, Fondue and other structural components and the enzyme transglutaminase, which crosslinks clot components and which also sequesters microbes to the clot (Scherfer, Karlsson et al. 2004; Lesch, Goto et al. 2007; Lindgren, Riazi et al. 2008). Sharing homology with clotting factor XIIIa, *Drosophila* transglutaminase is the only clot component with a clear orthologue in humans (Wang, Wilhelmsson et al. 2010). The key enzyme of the melanization cascade, PO also has a role in coagulation where it catalyses further crosslinking of the initial, primary soft clot that forms due to transglutaminase activity. Consequently, larvae that lack PO activity not only show reduced melanization at the wound site but also have wounding defects (Bidla, Lindgren et al. 2005).

### 1.2.7 The prophenoloxidase activating system (PAS)

The activation of prophenoloxidase (PPO) upon infection is a hallmark of arthropod immunity. Most arthropod POs carry out tyrosinase reactions, i.e. once activated, they catalyze the conversion of mono- and diphenols to orthoquinones that polymerize non-enzymatically to melanin. Some of the by-products of this reaction are cytotoxic and have been implicated in pathogen killing although this is controversial (Nappi and Vass 1993; Nappi, Vass et al. 1995). It is generally accepted that PAS is important during wound healing, sequestration of bacteria and encapsulation. Recent evidence also implies that PO activity is involved in supporting phagocytosis (Cerenius, Lee et al. 2008). The relevance of PAS in combating infection appears to be species and pathogen dependent. While crucial for host survival in many arthropod-pathogen encounters, melanization despite being activated seems to be less relevant for successful clearance of bacterial and fungal infection in *Drosophila melanogaster* (Leclerc, Pelte et al. 2006; Ayres and Schneider 2008). Unlike vertebrate tyrosinases arthropod POs are not integral membrane components confined to specific organelles, the melanosomes (Sanchez-Ferrer, Rodriguez-Lopez et al. 1995). Instead

they are released by specialized hemocytes into the surrounding medium, usually the hemolymph (Cerenius, Lee et al. 2008).

Surprisingly, despite being released, the vast majority of POs characterized so far lacks a signal peptide required for classical, endoplasmatic-reticulum mediated secretion. In *Drosophila*, PPO is released upon crystal cell rupture, a process that is JNK dependent and shares similarities with apoptosis (Bidla, Dushay et al. 2007).

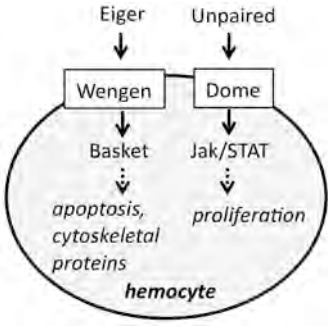


**Figure 4. Hemocyte activation pathways.** Eiger present in the hemolymph activates JNK (basket) signaling in hemocytes, which in turn triggers cytoskeletal remodeling or apoptosis dependent on the cell physiological context. Secreted cytokines of the unpaired family activate Jak/STAT supposedly via Domeless receptor leading to cell proliferation of hemocytes.

The proteolytic activation of PPO is tightly controlled since PO activity results in the production of cytotoxic substances and protein cross-linking. In many invertebrates, microbial products such as bacterial peptidoglycans and lipopolysacharides and fungal ß-1,3-glucans activate PPO through interaction with host recognition proteins (Cerenius and Soderhall 2004). This however remains to be shown in *Drosophila*. Moreover, host-derived molecules such as protein fragments of hemolymph proteins can trigger PPO activation in insects (Altincicek and Vilcinskas 2006). The number of PPO genes ranges between species from 10 in the mosquito *Aedes aegypti* to a single PPO encoding gene in the honey bee, *Apis mellifera*.

There are three genes coding for PPO in the fly genome: Monophenoloxidase, Diphenoloxidase A2, and Diphenoloxidase A3, the latter one is exclusively expressed in lamellocytes and might participate in melanization associated with encapsulation. Monophenoloxidase and diphenol oxidase A2 are expressed in crystal cells and are essential for any immunity- and wounding-associated melanization in *Drosophila*. Mutants that lack functional crystal cells such as *Black cells* ($Bc^1$), which contains aberrant, non-functional crystal cells also completely lack PO-activity in the

hemolymph (Rizki, Rizki et al. 1980; Sorrentino, Carton et al. 2002; Milchanowski, Henkenius et al. 2004; Jung, Evans et al. 2005).

After wounding and in response to certain infections, PPO is released into hemolymph by crystal cells. Activation of PPO is triggered during wounding and encapsulation. However, the exact mechanism by which activation is carried remains to be established. Data presented in this work suggest that ectopically induced apoptosis *in vivo* and membrane phospholipids *in vitro* have a role in PPO activation.

In *Drosophila*, a cascade of serine proteases controls PPO activation. The enzymatic cascade in turn can be inhibited by serpins, of which there are 29 encoded in the *Drosophila* genome. Three of them have been characterized so far (Spn27A, Spn28D and Necrotic). Spn27A has been shown to inhibit PPO activation in the hemolymph as a loss-of-function mutation of the gene results in formation of melanotic masses in the hemolymph (De Gregorio, Han et al. 2002). Its role is to restrict melanization at the wound site and to prevent systemic melanization. Serpin Spn28D seems to control initial release of PPO. Inactivation of Spn28D causes melanization of tissues in contact with air and pigmentation defects of the adult cuticle (Scherfer, Tang et al. 2008). Necrotic negatively regulates the Toll pathway by inhibiting the serine protease Persephone, which can directly activate Toll via Spätzle in the absence of microbial stimuli (Robertson, Belorgey et al. 2003; Pelte, Robertson et al. 2006). Loss of *necrotic* has pleiotropic effects including constitutive activation of Toll signaling and melanization.

Two serine proteases have been identified as PPO activating enzymes: Melanization Protease (MP) 1 and MP2. MP1 appears to be essential for melanization in response to both bacteria and fungi. The importance of MP2 (PAE1 or SP7) as a PPO activating enzyme (PAE) is debated: The work of Leclerc and coworkers defined the proteinase as the terminal PPO activating enzyme (Leclerc, Pelte et al. 2006) while another group found that MP2 was mostly relevant for fungi-triggered melanization but of minor importance in bacterial infections (Tang, Kambris et al. 2006). However, in a later study melanization in response to infection with a panel of bacterial strains was clearly affected in MP2 mutants (Ayres and Schneider 2008) and RNAi knockdown of the gene also blocked PO activity in fly extracts (Tang, Kambris et al. 2006).

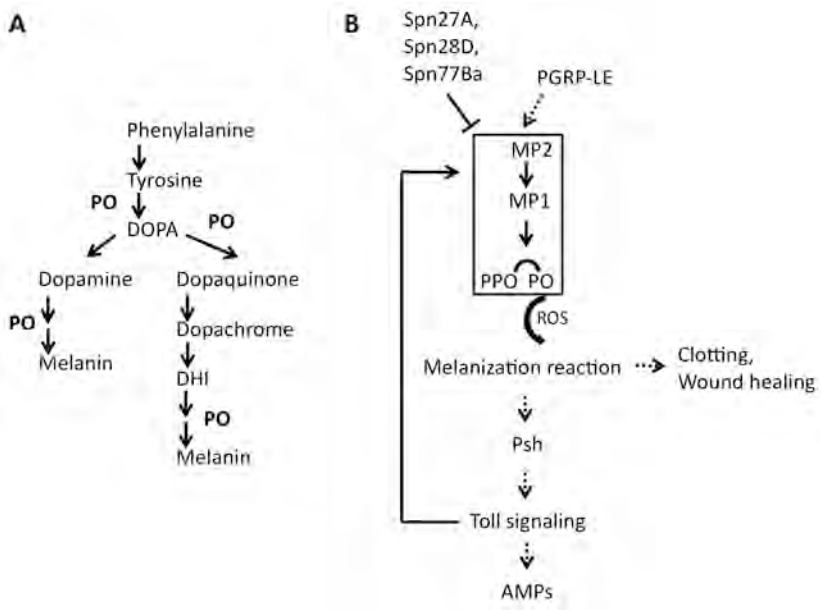


**Figure 5. (A) The role of phenoloxidase (PO) in Melanin biosynthesis in *Drosophila* - adapted from (Tang 2009).** Phenylalanine is hydroxylated to tyrosine, which is converted to DOPA by PO. DOPA is either catalyzed to Dopaquinone by PO or to Dopamine (by dopadecarboxylase, not shown). Dopamine reacts to Melanin via intermediates and PO catalysis. Dopaquinone can react non-enzymatically to Dopachrome, which is converted to DHI. PO catalysis leads to Melanin. (B) Known components of prophenoloxidase (PPO) activating cascade include two clip-domain proteases MP1 and MP2 and PPO. Known inhibitory serpins are Spn27A (hemolymph), Spn28D (hemolymph) and Spn77Ba (trachea). Melanin and its intermediates are important for clotting, wound healing and possibly pathogen killing. The melanin biosynthesis also activates Toll signaling via Persephone (Psh) resulting in AMP induction. Activated Toll in turn triggers melanization as does the DAP-type PGN recognition factor PGRP-LE. PPO activation. Core components of the PPO-activating serine protease cascade are MP2 and MP1. MP2 acts upstream of MP1, presumably not in a direct manner but via an unknown intermediate. MP1 putatively cleaves PPO. The melanization reaction leads to the production of ROS, which might play a role in pathogen killing and cytokine signaling. Melanization products contribute to clot formation and wound healing. Furthermore melanization products can activate Toll signaling via Psh, resulting in AMP production. Toll signaling itself also activates melanization as does PGRP-LE. Three serpins (Spn27A, Spn28D and Spn77Ba) have been demonstrated to block PPO activation.

## 1.3 Autoimmune mutants in *Drosophila*

Melanotic nodules or pseudo-tumors have been described in a number of *Drosophila* mutants. The prefix “pseudo” is used since in these mutants immune-targeted tissue despite forming tumor-like lumps never exhibits other characteristics of malignant vertebrate tumors such as tissue invasion and abnormal proliferation of cells. In fact, in many “pseudo-tumor” mutants the observed melanotic masses represent clumps of aberrant hemocytes only. They seem to be the result of either malfunctioning immune tissue or of apparently altered self that is recognized and targeted by hemocytes. Mutants, in which non-hematopoietic tissue is encapsulated by

plasmatocytes and lamellocytes are referred to as type 1 as opposed to type 2 mutants that are characterized by aberrant hemocyte numbers, which putatively aggregate and melanize (Watson, Johnson et al. 1991). In addition to these hemocyte-associated melanization phenotypes, mutants have been reported, in which tissues such as salivary glands, gut and tracheae are melanized but not encapsulated since hemocytes are not detected at the respective tissue (Minakhina and Steward 2006). In these mutants, the origin of PO-activity/ melanization is unclear.

## 1.4 The danger model to explain immune activation

Self non-self discrimination has long been considered a hallmark of immune activation. As also evident from studies in insects, immune responses have been studied mostly with regard to foreign, non-self insults. However, there is accumulating evidence that immune reactions are often triggered by endogenous, host-derived stimuli, which can become exposed to the immune system as a result of either external factors such as pathogen invasion or factors that are solely the result of genetic or epigenetic changes in the host (Matzinger 2002; Matzinger 2007).

### 1.4.1 The hydrophobicity model

Hydrophobic patches have been suggested and also demonstrated to serve as danger-associated patterns (DAMPs) that elicit immune responses (Seong and Matzinger 2004). It is well understood that pathogen and hence non-self derived molecules trigger immune responses through microbe-associated pattern (MAMP)-specific immune cell receptors. However, a growing list of self-derived, endogenous factors that are recognized by the same set of receptors clearly conflicts with this classical model of immune activation via self non-self discrimination. Conventional ligand-receptor binding involves van der Waal's forces, hydrogen bonding, ionic interactions and hydrophobic interactions to stabilize ligand-receptor pairing. However one feature that virtually all immune-stimulatory factors seem to share is spatially restricted hydrophobic patches. It has hence been proposed that many immune-activating receptors have evolved to simply recognize hydrophobicity or hydrophobic patches, respectively (Seong and Matzinger, 2004).

## 1.5 The concepts of cancer immunosurveillance and immunoediting

The idea that the immune system has evolved to recognize and to destroy aberrant tissue is termed immunosurveillance (Burnet 1970). The concept has originally been proposed in the 1950s (Burnet 1957), based on experimental evidence that mice could be immunized against tumor transplants. Studies in the 1990s showed that surveillance is dependent on interferon-gamma and lymphocytes (NK cells and T cells), both of which do not exist in *Drosophila.*

Already early in the 1970s it was speculated that the immune system might rather promote tumor-growth than inhibiting it. The theory was based on the idea that immune cells that recognize tumor antigens would provide a positive growth signal for the tumor. It is now accepted that the immune system can be both tumor-destructive and hence host-protective as well as tumor sculpting, and thereby tumor-growth promoting. This concept has been named (cancer-) immunoediting (for reviews on cancer immunoediting see: (Dunn, Bruce et al. 2002; Ostrand-Rosenberg 2008)). Cancer immunoediting comprises three steps: Elimination, Equilibrium and Escape.

Early during tumorigenesis, immune cells recognize and eliminate tumor cells. This step is called “elimination” and is equivalent to immunosurveillance. By attempting but not completely succeeding in tumor elimination it is believed that the immune system itself selects and promotes more competitive tumor cell variants, which will be unsusceptible against immune defense. This phase is recognized as “equilibrium”. Thus immune-sculptured tumor cells are able to escape from the original tumor (escape-phase). However, evidence for a contribution of innate immunity to the process of immunoediting is scarce.

## 1.6 *Drosophila melanogaster* as a cancer model

*Drosophila* mutants that lead to tissue overgrowth in larvae have been described as early as in the 1910s (Stark 1919). Work on cancer in *Drosophila* was pioneered already in the 1960s and 1970s when E. Gateff (Schneiderman and Gateff 1967; Gateff 1978) described mutants of genes, which when homozygous lead to significant overgrowth of various larval organs, i.e. imaginal discs, neural and hematopoietic

tissue. Isolated overgrown tissue from such tumor suppressor mutants could be maintained in adult host flies where it started to metastasize and invade distal sites of the host.

Out of the six hallmarks of cancer: 1. Continued cell proliferation: 2. Escape from apoptosis; 3. Loss of differentiation; 4. Invasion and metastasis; 5. Continued telomere elongation and 6. Sustained angiogenesis, the first four can be modeled in *Drosophila*.

Today, more than a dozen genes in *Drosophila* are known to function as tumor suppressors. In addition, a handful of oncogenes have been described, which lead to tissue over-proliferation when induced (Brumby and Richardson 2005; Vidal and Cagan 2006). Because of *Drosophila*'s open circulatory system mutations that are needed for angiogenesis are not required for tumor progression. Tumorous outgrowth is hence dependent on fewer mutations than in vertebrates.

## 1.7 Oncogenes and tumor suppressors

Tumors develop as a consequence of misregulation of cell proliferation and differentiation. Many cancer causing oncogenes are mutated versions of growth factors (a.k.a. mitogens) or components of growth factor signaling pathways. Contrary to oncogenes, many tumor suppressor genes code for proteins that negatively regulate cell cycling or induce apoptosis. Loss-of-function mutations of tumor suppressors can contribute to tumor development, or - at least in *Drosophila* - can be sufficient for tumorigenesis. One of the most prominent oncogenes is dominant-active Ras. The Ras GTPase links extracellular mitogens to intracellular pathways that coordinate cell proliferation, most notably the Mitogen-activated-protein-kinase (MAPK) pathway. Ras acts as a molecular switch, that means it becomes active upon GTP binding and inactive when GTP is hydrolysed to GDP. Hydrolysis is dependent on an additional protein, GAP (GTPase activating protein). Hence, mutation in Ras that prevent GAP binding, will render Ras constitutively active (i.e. oncogenic) as the transition to the inactive GDP form is inhibited. Besides promoting proliferation, dominant-active Ras also blocks JNK dependent apoptosis.

### 1.7.1 Tumor suppressors of the apicobasal polarity module

In *Drosophila*, homozygous loss-of-function (LOF) mutations in *scribbled* (*scrib*), *discs large 1* (*dl1*), *lethal (2) giant larvae* (*l(2)gl*) lead to neoplastic tumors in epithelial larval tissue that fulfill the four hallmarks of cancer that can be modeled in *Drosophila* (Humbert, Grzeschik et al. 2008). Mammalian homologues of these genes exist (Lue, Marfatia et al. 1994; Dow, Brumby et al. 2003; Grifoni, Garoia et al. 2004) and have also been implicated in cancer as they are targeted by certain oncoviral proteins. *Scrib*, *dl1* and *l(2)gl* have been shown to regulate apicobasal polarity as well as cell proliferation and apoptosis (Humbert, Russell et al. 2003): In cancer, the loss of cell polarity is a frequently observed phenotype required for tumor cells to detach from its surrounding tissue. Apicobasal polarity is required for interaction of cells via septate junctions and interaction of cells with the extracellular matrix. Loss of these interactions is observed in mutants of the genes *scrib*, *dl1* and *l(2)gl*. Moreover, the Scribbled polarity module negatively regulates cell proliferation by inhibiting the cell cycle regulator Cyclin E and by promoting apoptosis through blocking expression of the apoptosis inhibitor dIAP1 (Yamanaka and Ohno 2008).

### 1.7.2 The Hippo tumor suppressor pathway

Other tumor suppressor genes that have been looked into in this work can be assigned to the Hippo tumor suppressor pathway (Menendez, Perez-Garijo et al. 2010; Zhao, Li et al. 2010). The involvement of this pathway in tumorigenesis has mostly been in studied in *Drosophila* yet components of the Hippo pathway are highly conserved in mammals and evidence from patient samples, mouse models and cancer cell lines indicates that the analogous pathway in humans is also involved in tumorigenesis (Zeng and Hong 2008). Like Scrib, Dl1 and L(2)gl the Hippo pathway is an important regulator of organ size by controlling both cell proliferation by targeting Cyclin E and promoting apoptosis via inhibtion of dIAP1. Deregulation of this pathway in *Drosophila* leads to a dramatic increase in organ size (Pan 2007; Zhang, Yue et al. 2009).

The core pathway is composed of two serine/threonine kinases, Hippo and Warts. The phosphorylation-mediated activation process of Hippo is not completely

understood but it involves the moesin-domain containing proteins Expanded and Merlin as well as the protocadherin Fat. Once activated, Hippo phosphorylates two scaffolding proteins and Warts. Activated Warts phosphorylates and thereby inhibits Yorkie, leading to downregulation of its targets dIAP1, Cyclin E and the microRNA Bantam (Zeng and Hong 2008). This results in a reduction of cell number and hence smaller organs. In contrast, inactivation of the Hippo pathway leads to an increase in organ size due to enhanced cell proliferation. For a reviews see (Vidal and Cagan 2006; Halder and Johnson 2011).

## 1.8 Tumour necrosis factor (TNF)

In mammals, proteins of the tumor necrosis factor (TNF) family are involved in the regulation of inflammation, autoimmune diseases and apoptosis (Chen and Goeddel 2002; Wajant, Pfizenmaier et al. 2003). TNF family members are ligands that can act membrane-bound or proteolytically cleaved as soluble cytokines in a paracrine, endocrine or in an autocrine fashion. The TNF receptors are membrane proteins that polymerize upon ligand-binding, resulting in recruitment of cytosolic proteins to their cytoplasmic domains. This leads to activation of cell death, NF-kappaB and JNK pathways.

### 1.8.1 TNF signaling and mammalian cancer

A role of TNF in mammalian tumor biology is well recognized (Balkwill 2009). Paradoxically TNF is able to both restrict and to promote tumor growth. In experimental cancer models it has been shown that TNF can induce cell death in tumors. Yet there are numerous cases known in the literature where TNF inhibition lead to decreased tumor growth. It has further been shown that NF-kappaB signaling links TNF and tumor progression. TNF produced by tumor cells, myeloid cells and supposedly other cell types in the tumor microenvironment acts primarily through TNFR1 in an autocrine and paracrine manner. Autocrine actions further increase malignancy of tumor cells, which in turn result in secretion of other cytokines that act on immune cells such as tumor-associated macrophages (Mantovani and Sica 2010).

The pro-tumor actions of TNF have been shown in a variety of experimental tumors and human cancer biopsies and may be tumor and tissue specific.

In tissue culture studies, TNF was reported to be specifically toxic to malignant cells. However, it seems to be only cytotoxic to cells in the presence of metabolic inhibitors when the default cell survival and inflammatory pathways downstream of TNF signaling are inactivated, hence apoptosis induction can proceed.

Natural killer (NK) cells and other myeoloid lineage immune cells from TNF knockout mice exhibit impaired cytotoxic activity and TNFRs have been implicated in tumour surveillance. In summary, there is evidence for both pro- and anti-tumour activity of TNF in mammals.

In flies, the only TNF homologue is Eiger, which together with its receptor Wengen constitute a simple TNF-like signaling system. Both Eiger and Wengen are expressed during embryogenesis and Eiger is responsive to genotoxic stress. Ectopic expression of either Eiger or Wengen causes JNK signaling dependent cell death. For instance, the expression of Eiger induces apoptosis via JNK signaling in cells that carry a loss-of-function mutant of the tumor suppressor gene *scribbled* (Igaki, Kanda et al. 2002; Kanda, Igaki et al. 2002; Igaki, Pastor-Pareja et al. 2009). However, dependent on the physiological context, Eiger may also promote tumorigenesis presumably via inducing matrix metalloproteinase (see paragraph 1.9) in the tumor (Cordero, Macagno et al. 2010).

## 1.9 Breakdown of extracellular matrix is required for tumor invasiveness

The extracellular matrix (ECM) including the basement membrane represents a barrier to cell migration, which has to be degraded to promote invasion. It consists of structural components and signaling molecules, including collagen IV (Viking in *Drosophila*). Tumors often express proteolytic enzymes, namely matrix metalloproteinases (MMPs) that break down the ECM. *Drosophila* encodes two MMPs, MMP1, which has been implicated in metastasis of epithelial tissue, and MMP2, which has a function in tissue histolysis at metamorphosis (Page-McCaw 2008).

## 1.10 Apoptosis

Apoptosis or programmed cell death occurs in all multicellular organisms as a part of normal development. Cells that undergo apoptosis are characterized by morphological changes such as nuclear fragmentation, chromatin condensation and DNA fragmentation; loss of membrane asymmetry, cell shrinkage and cell blebbing (Saraste and Pulkki 2000). Apoptosis can be triggered by a range of endogenous and exogenous stimuli such as cytokines, cytochrome C and different types of cellular stress (e.g. radiation and hypoxia). Defined signaling pathways such as the JNK pathway regulate apoptosis. The removal of apoptotic corpses is highly controlled by a redundant system of receptors, bridging molecules and 'eat me' signals (Grimsley and Ravichandran 2003).

In *Drosophila*, three proapoptotic proteins, Head involution defective (Hid), Grim and Reaper regulate cell death by interaction with Inhibitor of apoptosis protein 1 (dIAP1) (Steller 2008). In the absence of the three proteins, apoptosis is virtually completely blocked. In contrast, ectopic expression of either of these genes leads to apoptosis. Despite sharing little overall sequence similarity, the three proteins share one common motif, IBM (IAP-binding motif), which is required for interaction with IAP and cell death. IAPs in turn inhibit caspases, which are the essential core enzymes of apoptosis.

## 1.11 Advantages of *Drosophila melanogaster* as a model system

The fruitfly offers several advantages over vertebrate model systems such as mice to address questions relevant for multicellular organisms. Besides shorter generation time and greater fecundity, *Drosophila* is cheaper to maintain and the degree of genetic tractability/ manipulation is unmatched in other multicellular model systems. Public stock collections provide mutant fly strains that cover most genes of the *Drosophila* genome. Transgenes can be expressed in a temporal and tissue/cell specific manner and they can like-wise also be knocked-down by expression of RNAi-constructs that are available for almost 90 % of all *Drosophila* genes (http://www.vdrc.at/rnai-library).

## 1.12 Outline of this project

The relevance of endogenous stimuli and foreign elicitors in immune activation will be assessed in a comparative study between Lepidopteran and Dipteran species. The immune response will be measured as PO-catalyzed melanization.
Moreover, aberrant cell fate will be induced genetically in *Drosophila* in non-immune tissue and the immune response to this altered tissue investigated. Ultimately, the functional consequences of the interplay between immune system and altered self will be studied. Altered self will be induced by knocking down tumor suppressor genes and by expressing a dominant-active form of Ras oncogene at 85D ($Ras^{V12}$), which is the *Drosophila* homologue to the vertebrate proto-oncogene H-Ras. Hence, conversely to the first study, the genetic determinant of altered self will be known. The development of aberrant tissue and the immune response against it will be characterized by means of immunohistochemistry, genetics and image analysis.

The yeast GAL4 system will be applied for induction of aberrant tissue. Immune responses will be studied by means of histology, photometry, immunocytochemistry and genetic mutants (epistatic interactions).

# 2 Materials

## 2.1 Reagents

Bovine serum albumin (BSA) (Sigma-Aldrich)

Formaldehyde 37% (Sigma-Aldrich)

1,4-diazabicyclo[2.2.2]octane (DABCO®, Sigma-Aldrich)

L - alpha-phosphatidylethanolamine (P-8068)

L -alpha-phosphatidylinositol sodium (P0639)

L -alpha-phosphatidylcholine (PC) (P3556)

L -alpha-phosphatidyl-L -serine (P0474)

All purchased from Sigma-Aldrich

Peptidoglycan (PGN) purified from *Bacillus megaterium* (kindly provided by Håkan Steiner, Stockholm University, Sweden)

Lipopolysaccharide from *Escherichia coli* O55:B5 (Sigma-Aldrich)

Finsprit 95% (Kemetyl)

Nipagin™ (H-5501, Sigma-Aldrich)

Glycerol (Sigma-Aldrich)

Antibodies and cytostains

Mouse-anti-H2 (Hemese) used in (Kurucz, Zettervall et al. 2003)

Mouse-anti-L1 (Kurucz, Vaczi et al. 2007)

Mouse-anti-L2 (Kurucz, Vaczi et al. 2007)

Mouse-anti-Lozenge C-terminal protein (Developmental Studies Hybridoma Bank, University of Iowa)

Mouse-anti-PPO (gift from Michelle Crozatier)

Mouse-anti-dMMP1 (catalytic domain) (Developmental Studies Hybridoma Bank, University of Iowa)

Cy3-conjugated rabbit-anti-mouse (Jackson ImmunoResearch)

FITC-conjugated rabbit-anti-mouse (Jackson ImmunoResearch)

TRITC-conjugated Phalloidin (Sigma-Aldrich)

DAPI (4',6-diamidino-2-phenylindole) solution (Sigma-Aldrich)

Hoechst 33342 stain (supplied with Poly-caspase FAM-FLICA™ *in vitro* Apoptosis Detection Kit, Immunochemistry)
Propidium Iodide (Sigma-Aldrich or supplied with Poly-caspase FAM-FLICA™ *in vitro* Apoptosis Detection Kit, Immunochemistry)

## 2.2 Buffers and solutions

Phosphate buffered saline (PBS)

| | |
|---|---|
| NaCl | 137 mM |
| KCl | 2.7 mM |
| $Na_2HPO_4$ | 10 mM |
| $KH_2PO_4$ | 1.76 mM |

Adjusted to pH 7.4

*Drosophila* Ringer' saline

| | |
|---|---|
| 1 M $KH_2PO_4$ | 0.37 mM |
| 1 M $CaCl_2$ | 1.5 mM |
| 0.5 M $Na_2HPO_4$ | 2 mM |
| 1 M KCl | 5 mM |
| 5 M NaCl | 130 mM |

Bi-distilled water (milli-Q grade) was added to the final volume and the pH adjusted to 6. The buffer was sterile-filtered and stored at temperatures between RT and 4˚C.

*Galleria* Ringer's saline

1 M Tris-HCl
5 M NaCl
1 M KCl
1 M $MgCl_2$
1 M $CaCl_2$

Bi-distilled water (milli-Q grade) was added to the final volume and the pH adjusted to 6.8. The buffer was sterile-filtered and stored at temperatures between RT and 4˚C. For anti-coagulant Ringer' s saline (Scherfer, Karlsson et al. 2004) $CaCl_2$ was substituted by EDTA to prevent coagulation.

L-3,4-dihydroxyphenylalanine (L-DOPA) buffer

L-DOPA (Sigma-Aldrich) was solved in 10 mM sodium phosphate buffer (pH 6.6. at RT) under vigorous shaking and protected from light to yield a 20 mM L-DOPA solution. The buffer was always freshly prepared prior to use from 0.2M dibasic sodium phosphate (solution A) and 0.2M monobasic sodium phosphate (solution B). The solutions were mixed in appropriate ratios and filled up with deionized water of Milli-Q quality to yield a working buffer of pH 6.6.

solution A

0.2 M $NaH_2PO_4$

solution B

0.2 M $Na_2HPO_4$

3.7 % formaldehyde fixative

37 % formaldehyde (FA) was diluted 10x in PBS. The solution was always freshly prepared prior to use and discarded after 1 day.

Glycerol DABCO® mounting medium (70% glycerol, 2.5% DABCO)

35mL of glycerol was mixed with 15 ml PBS and 1.25 g DABCO crystals in a 50 ml plastic tube and the solution homogenized by vigorous shaking on a rocking platform. The solution was stored protected from light at room temperature.

## 2.3 Fly strains

RNAi lines from VDRC, Austria (Dietzl, Chen et al. 2007)

| CG number | Transformant ID | Name | Insertion on chromosome |
|---|---|---|---|
| CG2671 | 51247/GD | *lethal (2) giant larvae* | 3 |
| CG1725 | 41136/GD | *discs large 1* | 3 |
| CG5954 | 13994/GD | *lethal (3) malignant brain tumor* | 3 |
| CG1725 | 41134/GD | *discs large 1* | 2 |
| CG2671 | 51249/GD | *lethal (2) giant larvae* | 3 |
| CG3352 | 9396/GD | *fat* | 2 |
| CG5954 | 12709/GD | *lethal (3) malignant brain tumor* | 3 |
| CG12072 | 9928/GD | *warts* | 3 |
| CG5462 | 105412/KK | *scribbled* | 2 |
| CG5671 | 101475/KK | *Pten* | 2 |
| CG5671 | 35731/GD | *Pten* | 3 |
| CG33336 | 10692/GD | *p53* | 3 |
| CG33336 | 38235/GD | *p53* | 2 |
| CG42317 | 32877/GD | *C-terminal Src kinase* | 3 |
| CG4859 | 101505/KK | *Matrix metalloproteinase 1* | 2 |
| CG6281 | 15372/GD | *Tissue inhibitor of metalloproteases* | 3 |

Stocks from the Bloomington Stock Center (Indiana University, USA)

| ID | Fly base genotype | Additional information |
|---|---|---|
| 1795 | w*; P{GawB}30A/CyO | GAL4 in imaginal discs |
| 1782 | w*; P{GawB}32B | GAL4 in imaginal discs |
| 1747 | y1 w*; P{GawB}71B | GAL4 in imaginal discs |
| 9448 | w*; P{GawB}OK384/TM3, Sb1 | GAL4 in the antennal imaginal disc |
| 1878 | w*; P{GawB}T80/CyO | GAL4 expression ubiquitous in third instar imaginal discs |
| 3735 | P{GawB}c701b | GAL4 pattern in third instar larva: anterior region of wing, haltere, and leg discs, brain - throughout eye disc, medial segment of brain lobe |
| 8860 | P{GawB}BxMS1096 | *Bx$^{ms1096}$ GAL4* (salivary gland and wing imaginal disc specific) |
| 6357 | P{Lsp2-GAL4.H}3 | GAL4 expressed in third instar fat body |
| 6397 | P{Hml-GAL4.G}6-4 | GAL4 expressed in larval hemocytes |
| 5460 | P{GAL4-da.G32}UH1 | *Daughterless GAL4* (ubiquitous GAL4 expression) |
| 3954 | y1 w*; P{Act5C-GAL4}17bFO1/TM6B, Tb1 | *Actin5C GAL4* (ubiquitous GAL4 expression) |
| 7017 | w*; P{tubP-GAL80ts}2/TM2 | *Tubulin GAL80$^{ts}$* |
| 6870 | w1118; P{Sgs3-GAL4.PD}TP1 | Salivary gland specific GAL4 |
| 12200 | y1 w67c23; P{lacW}spagk12101/CyO | *Spaghetti$^{LOF}$* mutant (CG13570) |
| 4847 | w[1118]; P{w[+mC]=UAS-Ras85D.V12}TL1 | *UAS-Ras$^{V12}$* (CG9375) |
| 13494 | y[1]w[67c23]; P{y[+mDint2] w[BR.E.BR]=SUPor-P}Sp7[KG02818] ry[506] | *MP2$^{LOF}$* mutant (CG3066$^{KG02818}$) |
| unknown | w1118, y1 w*; P{UAS-mCD8::GFP.L}LL5 | GFP-tagged mouse transmembrane protein CD8, which labels the cell surface |
| unknown | Bc[1] fj[1] wt[1] | *Black cells $^{1}$* (*Bc$^{1}$*) |

Other strains

*Tep1- GFP* (GFP is expressed under a *tep1*-promoter, gift from Marie Meister)

*Hemese* (*He*) *GAL4 UAS-GFP* (Zettervall, Anderl et al. 2004)

*Hemese GAL4 tubulin (tub) GAL80$^{ts}$* (generated by Ines Anderl, Umeå University, Sweden)

*Lozenge* (*Lz*) *GAL4; UAS-GFP*

*Glass (GMR) GAL4* (P{GAL4-ninaE.GMR}12)

*5253 GAL4* (GAL4 expressed in fat body, gift from Mitch Dushay, Illinois Institute of Technology)

*5267 GAL4* (GAL4 expressed in fat body, gift from Mitch Dushay, Illinois Institute of Technology)

*Pumpless (ppl) GAL4* (GAL4 expressed in fat body, gift from Bruno Lemaitre, École Polytechnique Fédérale de Lausanne, Switzerland)

*egr*$^{3}$ (Igaki, Kanda et al. 2002)

*spn27A*$^{1}$/ CyO (gift from Bruno Lemaitre)

*UAS-Spn27A* (Ligoxygakis, Pelte et al. 2002)

*UAS-grim* (Wing, Schwartz et al. 2001)

*UAS-reaper* (Wing, Schwartz et al. 2001)

*UAS-head involution defective* (*hid*) (Wing, Zhou et al. 1998)

## 2.4 Consumables

50 mL and 15 mL plastic tubes (TPP)

Glassware (DURAN series, SCHOTT)

1.5 ml micro tubes (Plastibrand)

Diamond precision tips (Gilson)

Glass capillaries (outer diameter: 2.0, inner diameter: 0.5 mm, Harvard Apparatus)

Multi-well slides, 10-well, 4 mm diameter/ well (MP Biomedicals)

Microscope depression slides (VWR International)

Microscope slides (Super Grade, CITO CLAS)

Cover glasses: 13 mm diameter (VWR International) used for blood smears; for other preparations: 24 mm x 32 mm (Solveco)

Petri dishes (diameter: 9 cm, TPP)

Costar® EIA/RIA plates (96-well, half area, flat bottom, high binding, non-sterile, polystyrene, Corning Incorporated)

Bottle-Top Vacuum Filter System (250 ml, 0.22 µm polyethersulfone filter, Corning)

## 2.5 Tools and devices

Microscopes and accessories

MZ FL3 including a dual gooseneck fiber optic illuminator (Leica)

LSM 510 META (Zeiss)

Stemi 2000-C including a gooseneck illuminator (Zeiss)

Axioplan 2 equipped with a Fluo Arc tungsten light source and a rotatable stage and an AxioCam HRm camera (sensor size: 8.9 mm x 6.45 mm) (Zeiss)

Axioplan 2 equipped with a Fluo Arc tungsten light source (Zeiss) and an Orca-ER camera (sensor size: 8.67 mm x 6.6 mm) (Hammamatsu)

Objectives (Zeiss): Apochromat 20x, 10x and Neofluor 2.5x

Orthoplan

Camera COOLPIX 4500 (Nikon)

MiniSpin microcentrifuge (Eppendorf)

Vortex Genie 2 vortexer (Scientific Industries)

Q3A1 heating block (Grant)

DuoBath water bath (Clifton)

DUOMAX 1030 rocking table (Heidolph Instruments)

Incubators (ASSAB Medicin AB and Friocell, Medcenter Einrichtungen GmbH)

custom-made injector (Stockholm University)

Needle puller (Narishige Scientific Instrument Lab)

Vmax™ Kinetic Microplate Reader (Molecular Devices)

Magnetic Stirrer (IKA Labortechnik)

HF-2000G Scale (Labassco)

AE 260 DeltaRange® precision scale (METTLER)

inoLab pH meter (WTW)

Q-POD® water purification system (Millipore)
Hot plate (Kervel)
Microwave oven (various manufacturers)

Precision dissection tweezers (Dumont, type 5)
Micropipettes with maximum volume ranging from 10 µL to 5 mL (Finnpipette)
Thermometer (various manufacturers)

## 2.6 Software

imageJ for Mac™ (http://rsbweb.nih.gov/ij/)
AxioVision™ software package (release 4.6.3 including modules for Z-stack recording and extended focus, Zeiss)
ANOVA (http://faculty.vassar.edu/lowry/anova1u.html)
Excel 2008 for Mac™ (Microsoft)
Powerpoint 2008 for Mac™ (Microsoft)

# 3 Methods

Unless otherwise noted experiments have been carried out at room temperature (RT), i.e. at a temperature varying between 20 – 22 ˚C. Fine dissection forceps (DUMONT, type Nr. 5) were used in all experimental steps that require the use of forceps, as further specified in the following protocols. Specific details about the reagents, tools or devices are mentioned in the text if necessary to avoid ambiguity.

## 3.1 Insect culture

### 3.1.1 *Drosophila melanogaster* culture

*Drosophila melanogaster* was kept at 25°C on a 12h light/dark cycle and reared on potato sucrose medium in plastic vials or bottles depending on the number of flies/ larvae needed for experimental procedures.

For the preparation of 1 L *Drosophila* food, 1.1 L of tab water was brought to a boil. Dry yeast, syrup, instant mashed potato and agar were added under vigorous stirring. The blend was boiled up again for 15 min and then allowed to cool down to 60-70 °C. Clumping of ingredients was avoided by stirring. Nipagin(TM) stock (concentration: 50 g / L) and ascorbate were then added. The blend was constantly stirred for homogenization and further cooling to about 50 °C. To avoid solidification food was portioned into vials and bottles thereafter. Filled, open vials and bottles were cooled down in an airstream for about an hour to speed up solidification and to keep flies away. A few crumbs of dry yeast were added to vials prior to use to promote egg laying.

Potato sucrose medium

Ingredients / L

12.9 g dried yeast (Santa Maria)

50 ml corn syrup (DanSukker)

40 g instant mashed potato (Marlin Olsson)

10 g Agar Agar (AB Ragnar Lundberg)

8.5 ml Nipagin(TM) (Sigma-Aldrich)
0.25 g ascorbate (Sigma-Aldrich)

### 3.1.2 *Galleria mellonella* culture

*Galleria mellonella* was reared on specific artificial diet at 25˚C under a controlled photoperiod (12h light/dark cycle).

*Galleria* medium

The following ingredients were blended together:

100 g rice-based cereals (FRIGGS)
200 g wheat germs (Kungsörnen)
1 g dry yeast (Santa Maria)
0.15 g Nipagin(TM) (Sigma-Aldrich)
2 multi-vitamin capsules (TwinLab “Daily One”)

After reaching a homogenous mix, the remaining ingredients were added:
100 mL liquid honey (different producers)
100 mL glycerol (Sigma-Aldrich)

*Galleria* medium could be stored at 4 °C for several weeks and was allowed to adjust to room temperature before fed to *Galleria* cultures.

### 3.1.3 Rearing of *Pararge aegeria*

*Parage aegeria* was reared on *Dactylis glomerata* host plant at approximately 17°C on a 12 h light/dark cycle. A strain from Madeira was used that does not undergo diapause (gift from Karl Gotthard, Stockholm University, Sweden). Adults were maintained in flight cages with host plants randomly distributed for egg laying. Individual plants with eggs were thereafter transferred to plastic cups with netting on top. Larvae were fed fresh plants when required.

### 3.1.4 Rearing of *Anopheles gambiae*

*Anopheles gambiae* was reared at 27 ± 2 °C and 70 ± 10% relative humidity on a 12-h light/12-h dark cycle (gift from Ingrid Faye, Stockholm University, Sweden). Eggs were laid on wet filter papers and transferred to water trays. Larvae were fed Tetramin® fish food. Adults were kept in cages made of cardboard or of steel with netting on top. Mosquitoes were offered 10 % sucrose–water solution *ad libitum*, and blood from a human volunteer once a week. The sugar–water solution was boiled and, after cooling, supplemented with Ampicillin (100 mg/ml) and Kanamycin (50 mg/ml).

## 3.2 Fly Handling

Flies were anesthetized with industrial grade $CO_2$. The $CO_2$ was stored in dedicated tanks that were connected to a flow regulator, which in turn allowed gas flow to a gas-permeable pad and to a needle that was inserted into vials/ bottles to anesthetize flies before transferring them to the pad. The flow of $CO_2$ was maintained to facilitate

inspection and handling of the flies using a fine paintbrush. Flies were inspected under a stereo dissection microscope (Zeiss Stemi 2000-C). When scoring for GFP, a dedicated adaptor with a lamp to excite GFP (Universal visualizing light source for fluorescent microscopy, Biological Laboratory Equipment, Maintenance and Service Ltd.) was mounted onto the microscope.

Female virgins were scored according to methods described previously (Greenspan 1997; Sullivan, Ashburner et al. 2000). A general rule is that females will almost certainly not except male flies until 10 – 12 hours after eclosion (at 18˚C). Briefly, virgin flies can be distinguished from older non-virgin flies by the lighter color of their cuticle, the inflated appearance of their abdomen and by a blackish-greenish spot that can be seen through the abdomen, the meconium. In addition, their wings are often still folded.

When setting up crosses to synthesize new genotypes preferentially five or more female virgins were mated with an equal or lower number of males.

GAL4 > UAS crosses were kept at specific temperatures ranging from 18˚C to 31˚C as indicated in the results section. To maintain temperatures higher than room temperature incubators were used (ASSAB Medicin AB; Medcenter Einrichtingen GmbH, Friocell). A water bath was used to heat up crosses to 31˚C.

### 3.3 Handling of larvae

Larvae were gently transferred with a fine paintbrush from vials or bottles to a weighing dish for washing in tab water at room temperature. Excess water on larvae was removed by allowing larvae to dry on tissue paper (KimWipes, Kimberly-Clark). For immobilizing larvae slides were prepared with scotch tape such that the sticky side was facing upwards. Larvae that were placed onto tape were immobilized but could be rinsed off easily with a drop of PBS or tab water.

### 3.4 Dissection of larval tissue

Dissection was performed in a depression slide with larvae immersed in PBS. Larvae were grabbed and hold gently with a pair of forceps at the anterior site. A second pair was used to carefully slice open the larva starting at the mouth hooks by pulling away the mouse part from the rest of the body. The respective tissue to dissect was identified based on morphology. Dissected tissue was stored in PBS on ice until further processing.

### 3.5 Dissection of wings

Wings were dissected with two pairs of tweezers and placed on microscope slides. Images of wings were taken against a light background with a Nikon COOLPIX 4500 camera mounted on a Leica MZ FL3 microscope. A dual gooseneck illuminator was used to ensure even illumination of the specimen.
The camera was adjusted to the maximum wide-angle position and the microscope set to 5x magnification. The white balance was set to incandescent. The aperture was set to a fixed value to allow for comparison between different specimens.

### 3.6 Hanging drop method

A humid incubation chamber was constructed consisting of a Tupperware™ box with inverted weighing dishes as slide rests inside. Humidity was maintained by placing water-soaked paper towels inside the chamber.
Five third instar wandering stage larvae were placed onto a multi-well slide (multi test slide, 15-well, MP Biomedicals) and opened from the middle with two pairs of modified dissection forceps: The forceps arms were slightly bent inwards to minimize loss of hemolymph through capillary forces. Larval hemolymph was collected in one well. During the procedure special care was taken to not injure external organs. Samples with visible tissue fragments were discarded. The slide was immediately inverted and placed into a humid chamber to allow clot formation at the interface between hemolymph and air (Figure 6). Samples were incubated for 30 min at 25 °C.

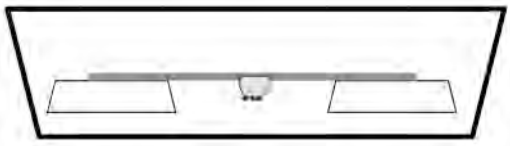

**Figure 6. Schematic drawing of a hanging drop preparation (not to scale).** The slide (grey line) with a drop of hemolymph (light grey) is inverted and rests on two weighing dishes (trapezoids) inside a Tupperware™ box (black line) to prevent evaporation. A clot forms at the interface between hemolymph and air (small circles).

### 3.7 Clot preparations

The clot was captured by carefully lowering a cover slip onto the drop surface. Preparations were transferred onto a drop of saline (*Drosophila* or Insect Ringer depending on the species) and analyzed under the microscope (Leitz Orthoplan). Images were taken with a Nikon COOLPIX 4500 camera adapted to the microscope. The camera was set to maximum focal length and white balance to incandescent.

### 3.8 PPO activation in hemolymph

To study melanization/ PPO activation morphologically, hanging drop preparations from *Galleria*, *Pararge*, *Drosophila* and *Anopheles* were performed as described in 3.6 and melanization analyzed under a Zeiss Stemi 2000 microscope. The magnification was set to 5x and images were taken with a Nikon COOLPIX 4500 adapted to the microscope. Even illumination of the specimens was achieved with a Dual Gooseneck Illuminator with both necks pointing towards the specimen. Hanging drop preparations to study PO activation were carried out at least three times per species showing basically the same melanization patterns. For comparison between *spn27A* depleted and wt samples L-DOPA buffer (see 2.2) was added to clot to reconstitute melanization.

Photometric measurements of PO activity were performed as described in (Bidla, Hauling et al. 2009). The protocol was based on work by (Sorrentino, Small et al. 2002). For *Drosophila*, between 12 and 15 larvae were quickly bled into a well of a 15-well slide and 7 µl of the hemolymph withdrawn. This volume was mixed on ice with 50 µl of *Drosophila* anti-coagulant Ringer' s solution. After pipette-mixing 5 µl of this hemolymph-suspension was aliquoted in triplicates to 1 µl of each phospholipid, peptidoglycan (PGN), lipopolysaccharide (LPS) (each suspended at a concentration of

10 µg/ml in 10 mM sodium phosphate buffer) and 10 mM sodium phosphate buffer (control). For *Galleria*, 4 drops of hemolymph from four last instar larvae were pooled on ice in 2 ml of *Galleria* anti-coagulant Ringer' s saline by amputating a pro-leg with a pair of forceps. After pipette mixing, 140 µl aliquots of the suspension were transferred in triplicates to 10 µl of elicitor suspensions and control buffer. Samples were allowed to activate for 10 min at 25 ˚C, thereafter 45 µl and 150 µl of 10 mM L-DOPA buffer were added to *Drosophila* and *Galleria* samples respectively, the samples transferred to 96-well microplates (Corning Incorporated) and the optical density (OD) was read at a wavelength of 490 nm with a Vmax™ Kinetic Microplate Reader (Molecular Devices). The OD measurement was repeated after incubating the samples for 30 min at 37 ˚C. Activation of PO was measured as the change in OD. Photometric measurements were repeated three times, showing the same trend.

### 3.8.1 Dot blotting

Dot blots have been carried out essentially as described previously (Sorrentino, Small et al. 2002): Hemolymph was collected from larvae and transferred onto Whatman filter paper that was soaked with L-DOPA buffer. Melanization was monitored and stopped by heating the incubated filter paper in a microwave oven at maximum power for approximately 10 sec. Images of dot blots were taken with a Nikon COOLPIX 4500 camera. For dot blots with elicitors hemolymph was collected from $w^{1118}$ larvae and aliquoted. 1 µl of elicitor suspension were added to individual aliquots and mixed. 1 µl of 10 mM sodium phosphate buffer was added to the control sample. All samples were processed in parallel and the experiment repeated at least three times, showing the same trend. For the comparison between bleeds form spn27A mutant larvae versus $w^{1118}$ (wt) hemolymph was collected from 5 larvae per sample and the same volume transferred to L-DOPA soaked filter paper and melanization followed as described above.

## 3.9 Injection of peptidoglycan (PGN) into larvae

Injection of PGN from *Bacillus megaterium* Bm11 was carried out using an in-house made microinjector, which facilitates pneumatic control of the injection volume.

Injection needles were drawn from glass capillaries with a custom-made micropipet puller. Larvae were immobilized using a brush (*Drosophila*) or hold with fingers (*Galleria*) for injection. PGN suspended in distilled water was injected at a concentration of approximately 2.5 mg/ml into the abdominal region. As a control, distilled water was injected. Larvae were then kept inside Petri dishes (diameter: 9 cm) at room temperature (22˚C) and melanization monitored for up to two hours. *Drosophila* and *Galleria* larvae were processed in parallel for each experiment. Experiments were repeated three times essentially with the same result.

## 3.10 Live imaging of GFP expressing larvae

Larvae were prepared as described in paragraph 3.3. Microscope slides were prepared with Scotch tape with the sticky side facing upwards. At both ends the stripe was inverted so that the inverted part would stick to the glass slide.

Dry larvae were transferred with tweezers onto the prepared slide and thereby immobilized. Images of whole larvae were taken under a Zeiss Axioplan 2 microscope using a Neofluor 2.5x objective and an AxioCam HRm camera. Larvae were illuminated by a tungsten lamp. To facilitate comparisons between specimens exposure time was fixed during imaging. The microscope was controlled by AxioVision Software (Zeiss).

In addition, to confirm that it was solely the fat body that expressed GFP, larvae were opened immersed in PBS with sets of forceps on a depression slide. A GFP-adaptor that was mounted to the stereomicroscope (Zeiss Stemi 2000-C) was used to excite the fluorophor. In the case of $Ras^{V12}$ and control tep1-GFP larvae images were taken with a Zeiss Axioplan 2 microscope using a 10x objective.

## 3.11 Immunocytochemistry and histochemistry

### 3.11.1 Detection of hemocytes and MMP1 on larval tissue

3rd instar larvae were briefly washed in tab water and excess water soaked on tissue paper. Larvae were opened in PBS from the anterior side using two pairs of dissection forceps. Opened larvae were briefly rinsed in PBS then fixed in 3.7 % formaldehyde

(prior to use diluted in PBS from 37 % stock) for at least 2 hours at room temperature or over night at 4 ˚C. Fixed tissue (salivary glands, fat body, imaginal wing disc) was dissected in PBS with two pairs of dissection forceps and stored in PBS. The dissected tissue was washed three times in PBS. To prevent unspecific antibody binding to surfaces the samples were blocked in PBS with 1% w/v BSA for 30 min to 1 h. Hemocytes were then labeled with Hemese-specific antibody (H2, diluted 1:5 in PBS 1% w/v BSA). Tissue was washed in PBS. The primary mouse-anti-H2 antibody was detected with fluorophor-conjugated goat anti-mouse antibody. (As fluorophors either Cy3 (1:200) or FITC (1:200) was used, depending on counterstaining of the tissue looked at.) For preparation for confocal microscopy samples were stained with TRITC-conjugated Phalloidin (1: 200) to stain filamentous actin (F-actin) in all cells. This step was omitted in GFP-expressing tissue as GFP fluorescence was preserved during specimen preparation. DNA was stained with DAPI (Sigma-Aldrich) diluted 1:5000 from stock. The specimens were washed in PBS twice and then mounted in glycerol-DABCO to delay fading. Cover slips were sealed with nail polish to prevent evaporation of the embedding medium. Slides were stored in the dark at temperatures between 4˚C and 8˚C until use. For detection of lamellocytes L2 antibody was diluted 50-fold in PBS + 1% (w/v) BSA and otherwise the protocol above followed. For detection of crystal cells antibody raised against the crystal cell specific transcription factor Lozenge (Lz) and Prophenoloxidase (PPO) were used. Lz-antibody was applied in a 50-fold dilution as for L2; PPO was used at 200-fold dilution. Specimen were permeabilized in PBS + 0.5 % (v/v) Triton-X 100 after fixation in two steps for 5 min each. Except for this alteration the same protocol was followed as for detection of H2 and L2. To preliminary assess whether hemocytes have adhered to salivary glands, nuclei were stained with DAPI. Nuclei for hemocytes can be distinguished from those of salivary gland cells due to their smaller diameter (Figure 7, B). Antibodies for all three classes of hemocytes were tested on blood smears from larvae (Figure 3, A – C). In addition, a *tep1*-GFP reporter was confirmed to be applicable as a marker for hemocyte detection on tumors (Figure 3, D): $Ras^{V12}$ salivary glands were dissected from larvae expressing $Ras^{V12}$ under $Bx^{ms1096}$ *GAL4* in a *tep1-GFP* background ($Bx^{ms1096}$ *GAL4* > *tep1-GFP;UAS-*$Ras^{V12}$) and either directly transferred to a drop of PBS on a microscopic slide and imaged under an epifluorescence microscope or briefly fixed and then stained with H2 antibody and

Cy3-conjugated goat anti-mouse antibody basically as described above. Dissected glands were fixed in 3.7% FA solution for 20 min and washed three times in PBS. Mouse - H2 antibody was used undiluted and allowed to bind to Hemese antigen for 2 h. Specimens were washed three times in PBS. Cy3-conjugated goat-anti-mouse antibody was added diluted 1: 100 in PBS + 1% (w/v) BSA and incubated with the sample for 1 h. After one washing step, DAPI (diluted 1:5000 in PBS) was added to the sample and incubated 10 min. The sample was washed three times in PBS and mounted in DABCO solution on a microscope slide for imaging.

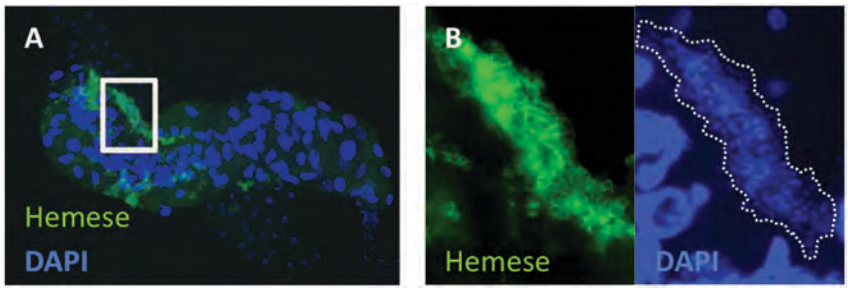


**Figure 7. DAPI as a hemocyte marker.** (A) Epifluorescence image of a $Ras^{V12}$ salivary gland. Hemocyte-specific Hemese antibody raised in mouse was detected with FITC-conjugated anti-mouse antibody. DAPI was used to stain DNA. Shown is a merged view of the two channels. The white rectangle marks the detail magnified in (B). Small DAPI-stained nuclei can be distinguished from salivary gland nuclei (area enclosed by dotted line) and correlate with Hemese-positive hemocytes.

For detection of MMP1, mouse anti-MMP1 antibody was diluted 1:50 in PBS + 1% (w/v) BSA. Tissue was prepared and immunostaining carried out as described for H2 and L2 antibody.

### 3.11.2 Detection of hemocyte classes in bleeds

Larvae were prepared as described. Larvae were bled individually into droplets of PBS on individual round cover slips (13 mm in diameter, VWR International). Cover slips were covered by placing them into a petry dish to prevent contamination and to impair evaporation of buffer-hemolymph suspension. Hemocytes were allowed to attach to the glass surface for 30 min. Excess buffer was soaked off using 200 µl pipette tips. Each sample was washed once then fixed in 3.7% FA for 10 min at RT. Fixative was pipetted off and samples washed 3 times. For lamellocytes detection, samples were initially stained with L2 antibody. Since lamellocytes can clearly be distinguished morphologically from other hemocytes. This step was omitted later on.

All samples were DAPI stained for 10 min at RT. Cover slips were mounted onto individual droplets of DABCO on microscope slides. No more than five cover slips were mounted per slide. Cover slips were sealed with nail polish and stored at 4˚C until microscopic analysis. Bleeds from larvae were scanned through manually using phase contrast at 20x magnification. Bleeds were classified according to abundance of lamellocytes into three classes (0 lamellocytes; 1-5 lamellocytes; >10 lamellocytes).
A more precise method to count lamellocytes using a Bürker chamber as described previously (Zettervall, Anderl et al. 2004) was discarded as lamellocyte numbers varied substantially between individual late 3$^{rd}$ instar larvae that express Ras$^{V12}$ in salivary glands and wing disc.

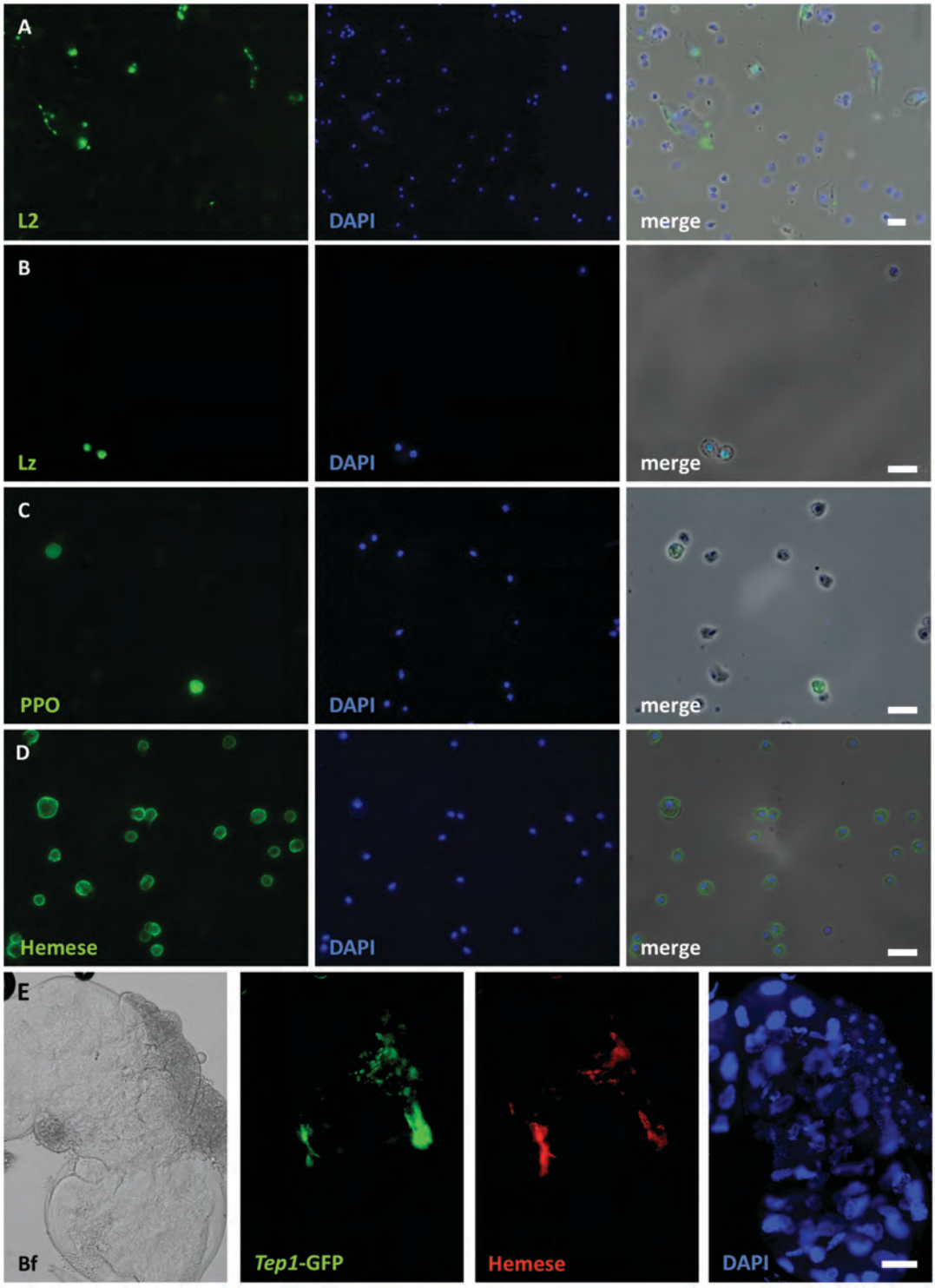


**Figure 8. Detection of hemocyte classes in blood smears.** (A) Epifluorescence images of a blood smear from a UAS-Ras$^{V12}$ larva. Lamellocytes are stained with mouse-L2 antibody detected by FITC-conjugated goat-anti-mouse antibody (L2). Nuclear DNA was stained with DAPI and both channels merged with the corresponding bright field image (merge). Crystal cells were detected with Lz-specific (B) and PPO-specific (C) antibodies that were raised in mouse. As in (A), the secondary antibody used was FITC-conjugated rabbit-anti-mouse. (D) Hemese-specific, pan-hemocyte antibody was detected as described for A – C. (E) In addition to the Hemese marker, hemoytes can also be detected due to their expression of a tep1 promoter controlled GFP reporter (Tep1-GFP). In this case Ras$^{V12}$ was induced in salivary glands (*Bx $^{ms1096}$-GAL4 > tep1-GFP;UAS-Ras$^{V12}$*). All images were taken with an epifluorescence microcope. Scalebars: 20 µm (A-D); 100 µm (E)

### 3.12 Detection of necrotic or late-apoptotic cell death in salivary glands

Larvae were prepared and glands dissected essentially as described in section 3.4 but special care was taken to not damage the tissue in order to avoid accidental necrosis due to handling. 1:5000 diluted propidium iodide (PI) was added to live glands and incubated for 10 min. Salivary glands were washed in PBS and subsequently fixed in 3.7 % FA for 40 min at RT. Fixative was washed out with PBS. Hemocytes were detected with H2-antibody (undiluted) by incubation for 1 h. FITC-conjugated goat anti-mouse antibody (1:200) was added to detect H2-antigen. Samples were washed in PBS and then mounted in DABCO solution. Cover slips were sealed and samples stored at 4 °C until microscopic analysis.

### 3.13 Detection of apoptosis in salivary glands

During apoptosis caspases become activated. Fluorescent inhibitors of caspases bind and label those caspases specifically. A kit based on these inhibitors was used (poly-caspase FAM-FLICA™ *in vitro* Apoptosis Detection Kit, Immunochemistry). FLICA™ reagent permeates cells where it will form covalent bonds with active caspases. Larvae were prepared and salivary glands dissected as described in (sections 3.3 and 3.11.1). Tissue was washed in PBS once. FLICA™ reagent was added to the samples and incubated for 60 min at RT to allow for reagent binding to active caspases. Samples were washed in PBS and fixed with a form-aldehyde-based fixative supplied in the kit (Figure 9). Hemocytes were detected as described in section 3.11.2 and mouse-Hemese-antibody detected with Cy3-conjugated goat anti-mouse antibody.

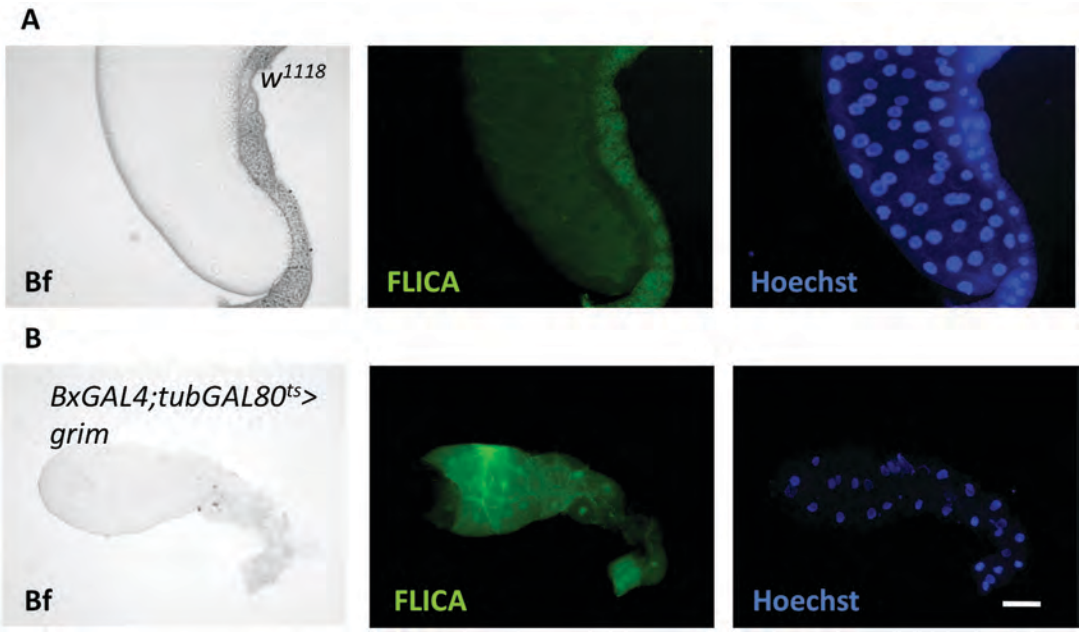


**Figure 9. FLICA staining to detect active caspases.** (A) Section of a wildtype (*w*$^{1118}$) salivary gland. Shown are: a bright field (BF) projection, FLICA staining to detect active caspases and Hoechst staining to label nuclear DNA. (B) Shown is a salivary gland in which apoptosis was induced by expression of the pro-apoptotic protein grim (*Bx-GAL4; tub-GAL80*$^{ts}$ *UAS-grim*). FLICA signal can be detected. An epifluorescence microscope was used. Scale bar: 100 µm.

## 3.14 Image analysis based quantification

### 3.14.1 Determination of salivary gland size

Images of whole glands were taken in brightfield mode using a Neofluor 2.5x objective and an AxioCam HRm camera. Images were saved in JPEG format for further use in image analysis software (imageJ, http://rsbweb.nih.gov/ij/). The total area per gland was measured in pixels by masking each gland using the "freehand tool" (Figure 10, A and B). The average gland size per sample (e.g. wt salivary glands at 25 ˚C) was determined by calculating the mean of all glands measured. At least 13 salivary glands were analyzed per group.

### 3.14.2 Quantification of hemocyte adherance on salivary glands

Hemocytes on salivary glands were FITC labeled and nuclei were DAPI-stained as described in 3.11.1. Prepared glands were mounted in DABCO to prevent fading. Images of whole glands were taken with an Apochromat 10x objective using an AxioCam HRm camera. This combination allowed to record immunofluorescence of

whole glands. I used a Zeiss microscope equipped with a rotatable stage. The microscope was controlled by the AxioVision™ software package (version 4.6.3 including modules for Z-stack recording and extended focus). To facilitate images of whole glands the stage was rotated when necessary. To detect FITC labeled hemocytes image stacks were recorded (100 ms exposure time, sections of 1.8 µm thickness) and extended-focus-images calculated from these stacks (wavelets method, no alignment). The resulting image was saved in JPEG format. Hemocytes on glands can be clearly distinguished from background due to their approximate circular shape and were marked (Figure 10, C and D) and their area measured in pixels in imageJ (http://rsbweb.nih.gov/ij/). Area values were measured per individual gland. The mean of all glands measured per sample (e.g. $Ras^{V12}$ glands at 29 ˚C) was divided by the average gland size of that sample. Since hemocyte numbers in *Drosophila* larvae are not normally distributed but rather follow a log-normal distribution (Sorrentino 2010), all area values were log-transformed (LN command in Microsoft Excel for Mac 2008). These values were plotted and standard deviation was calculated based on these values (Microsoft Excel for Mac 2008). At least 11 salivary glands were analyzed per group.

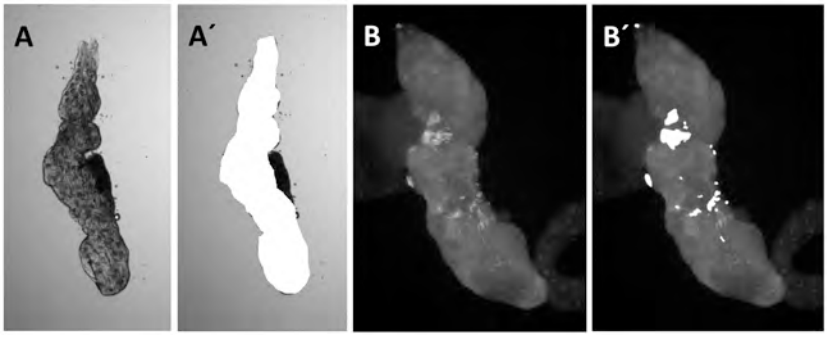


**Figure 10. Quantification of salivary gland size and hemocyte adherence.** Projection images of salivary glands (here: a wt gland) have been recorded in brightfield mode (A) and analyzed in imageJ: (A´) The freehand tool was used to mask the area occupied by the gland (white area). (B) primary mouse anti-Hemese-antibody has been detected with FITC conjugated goat-anti-mouse antibody and image stacks were recorded of whole glands (here: a $Ras^{V12}$ induced gland) and extended focus images calculated that were used for further analysis in imageJ. The area covered by hemocytes was masked in imageJ by drawing a line around the respective areas using the freehand tool (B´).

### 3.15 Statistical analysis

Samples from photometric measurements of PPO activation (see paragraph 3.8) and samples from imageJ measurements as described in sections 3.14.1 and 3.14.2 were compared and analyzed for significant differences in ANOVA (http://faculty.vassar.edu/lowry/anova1u.html) and by performing pairwise comparisons with a student T test (STT *command* in Microsoft Office for Mac 2008).

### 3.16 Ectopic induction of apoptosis and imaging

Apoptosis in hemocytes was induced by driving *UAS-grim* under *Hemese GAL4* or *Hemese GAL4 tubulin GAL80$^{ts}$*. For *Hemese GAL4* > *UAS-grim*, crosses were kept at 25˚C. For *Hemese GAL4 tubulin GAL80$^{ts}$* > *UAS-grim*, offspring larvae were kept at 25˚C until 2$^{nd}$ instar (about 3 days after egg laying), then transferred to 31˚C overnight and subsequently placed at 29˚C. Images of larvae with melanotic masses were taken with a Nikon COOLPIX 4500 camera mounted to a Leica MZ FL3 microscope including a dual gooseneck fiber optic illuminator.

### 3.17 Induction of aberrant self with the GAL4 system

To induce aberrant self a driver was crossed to respective UAS-tumor suppressor RNAi responder lines or to a UAS-*Ras$^{V12}$* responder strain. Crosses were kept at temperatures ranging between 18˚C to 29˚C as indicated in the result section.

<u>The yeast GAL4 system</u>

GAL4 is a transcriptional activator isolated from yeast. The target sequence of GAL4 is a 17-mer, referred to as UAS. Crucially, GAL4 is able to regulate transcription from the UAS site in organisms other than yeast including *Drosophila*.

The GAL 4 system is based on a transgenic driver line that expresses the transcriptional activator GAL4 and a responder line that contains a UAS-dependant transgene. Expression of the transgene is achieved by combining the two lines in a genetic cross. GAL4 expression depends on regulatory sequences adjacent to the integration site of the vector. Since GAL 4 is under a weak P transposase promotor its

temporal and spatial expression is dependant on endogenous enhancers. Over the years, a number of driver strains has been generated by integrating the GAL4 vector into the fly genome. Likewise, there are numerous UAS-responder lines available either from stock centers or shared between laboratories.

GAL4 activity is temperature-dependent being highest at around 30˚C and decreased at lower temperatures (Staehling-Hampton, Jackson et al. 1994; Wilder and Perrimon 1995). The GAL4 system can also be inhibited by crossing in a temperature-sensitive inhibitor termed GAL80$^{ts}$ that inactivates GAL4 (albeit often only partially) when the cross is kept below the permissive temperature (TARGET system, (McGuire, Le et al. 2003)).

The TARGET system

The regulate temporal expression of a UAS-responder can be regulated by making use of a temperature-sensitive variant of the yeast protein GAL80 (GAL80$^{ts}$), which binds to the transactivation domain of GAL4 thereby inhibiting its activity. Expression of GAL80$^{ts}$ itself is driven by a Tubulin (*tub*) promotor. By combining GAL4 driver, UAS-responder and *tub*-GAL80$^{ts}$ in one cross, transgenes downstream of UAS will be inactive at low temperature (18˚C) as GAL80$^{ts}$ binds GAL4 but this inhibition will be released upon shift to higher temperatures. A complete loss of GAL4 suppression is achieved at about 31˚C and maintained if crosses are subsequently shifted to 29˚C. However, partial loss of inhibition can be reached already at lower temperature, i.e. approx. 25˚C.

### 3.18 RNA interference (RNAi) as a tool to study gene function

In *Drosophila* genes can be silenced in a temporal and tissue/ cell specific manner. Essentially, silencing is mediated via expression of gene-specific inverted repeats in antisense direction that are under the control of UAS binding sites. GAL4 driven expression of such RNAi constructs results in formation of double stranded RNA-oligomers that are processed by Dicer into siRNAs, which direct sequence-specific degradation of target transcripts (Dietzl, Chen et al. 2007).

### 3.19 Generating fly strains

Since the matter of this work is the immune response to endogenous stimuli, i.e. factors that are encoded by the host genome, it is time saving to have an efficient strategy for combining transgenes, which are required for the induction of altered self (GAL4 driver and UAS responder oncogene/ UAS tumor suppressor RNAi construct) and immune deficiency mutants or responder lines that alter the immune stimulus. *Bx$^{ms1096}$ GAL4* has proven to be a particularly useful driver in this regard since the transgene is integrated on the X chromosome, allowing to generate strains that carry *Bx$^{ms1096}$ GAL4* and balancer on the second or third chromosome plus respective marker mutations on the respective corresponding chromosomes. Starting with these strains *Bx$^{ms1096}$ GAL4* can quickly be combined with UAS-responder transgenes and mutations on the 2nd or 3rd chromosome since only two crosses are required to generate the respective fly (plus a third generation to create flies that are homozygous for both GAL4 and the transgene/ mutation). In addition, the use of double balancer strains that often have poor survival/ fecundity can be avoided.

Example crosses to combine UAS-GFP.nls (homozygous on 3rd chromosome) with *Bx$^{ms1096}$ GAL4*:

*Bx$^{ms1096}$ GAL4*; *Dr*/Tm3 *Sb* was generated: *Bx$^{ms1096}$ GAL4* female virgins were crossed to *Dr*/Tm3 *Sb* males. From the offspring *Bx$^{ms1096}$ GAL4*; +/Tm3 *Sb* and *Bx$^{ms1096}$ GAL4*; +/*Dr* male flies were collected. Two crosses were set up: *Bx$^{ms1096}$ GAL4* female virgins crossed to *Bx$^{ms1096}$ GAL4*; +/Tm3 *Sb* males (cross A) and *Bx$^{ms1096}$ GAL4* females virgins crossed to *Bx$^{ms1096}$ GAL4*; +/*Dr* males (cross B). From cross A, *Bx$^{ms1096}$ GAL4*; +/Tm3 *Sb* female virgins were collected and crossed to *Bx$^{ms1096}$ GAL4*; +/*Dr* males from cross B. From the progeny, *Bx$^{ms1096}$ GAL4*; *Dr*/Tm3 *Sb* female virgins and males were selected and crossed to establish the stock.

*Bx$^{ms1096}$ GAL4*; *Dr*/Tm3 *Sb* female virgins were crossed to *UAS-GFP.nls*. *Bx$^{ms1096}$ GAL4*; *UAS-GFP*/Tm3 *Sb* offspring was collected (*Dr* negative and *Sb* positive) and respective female virgins and male flies crossed to establish the stock.

Balancers

Chromosomes that contain multiple invertions thereby suppressing meiotic recombination are referred to as balancer chromosomes or short: balancers. Furthermore, balancers carry dominant mutations that can easily be scored for as they produce a visible phenotype in adult flies and/ or larvae.

### 3.20 Fly stocks generated for this work

*Bx$^{Ms1096}$-GAL4*; *Sco*/CyO

*Bx$^{Ms1096}$-GAL4*; *Vno*/ TM3 *Sb*

*Bx$^{Ms1096}$-GAL4*; *UAS-GFP*

*Bx$^{Ms1096}$-GAL4*; *tubulin-GAL80$^{ts}$*

*Bx$^{Ms1096}$-GAL4*; *Bc$^{1}$*

*Bx$^{Ms1096}$-GAL4*; *egr$^{3}$*

*Bx$^{Ms1096}$-GAL4*; *wengen-RNAi*

*Bx$^{Ms1096}$-GAL4*; *UAS-Rab5$^{DN}$*/ Tm6 *Tb*

*Bx$^{Ms1096}$-GAL4*; *UAS-dicer1*

*5353-GAL4*; *UAS-GFP*

*UAS-GFP*;*UAS-Ras$^{V12}$*

*Bc$^{1}$*; *UAS-Ras$^{V12}$*

*UAS-scrib-RNAi*; *UAS-Ras$^{V12}$*

*Tep1-GFP*; *UAS-Ras$^{V12}$*

*Tep1-GFP*; *UAS-grim*

*Tep1-GFP*; *UAS-l(2)gl-RNAi*

*Tep1-GFP*; *sgs3-GAL4*

*Tep1-GFP*; *UAS-p35*

*Tep1-GFP*; *spag $^{k12101}$*/SM1

*spag $^{k12101}$*/CyO *Act5-GFP*

*spag $^{k12101}$*/CyO; *He-GAL4 UAS-GFP*

### 3.21 Fly stocks generated that will be used to address additional questions

*Bx$^{Ms1096}$-GAL4*; *eiger-RNAi* (KK 108814)/ CyO *Act GFP*

*Bx$^{Ms1096}$-GAL4*; *MMP1-RNAi* (KK 101505)/ CyO *Act* GFP

*Bx$^{Ms1096}$-GAL4*; *TIMP-RNAi* (GD 15372)

*Bx$^{Ms1096}$-GAL4*; *Hml$^{f033079}$*/ Tm3 *Sb*

*Bx$^{Ms1096}$-GAL4*; *MP1*$^{Def}$/ Tm3 *Act GFP*

*Bx$^{Ms1096}$-GAL4*; *CG3066$^{KG02818}$*/ Tm3 *Act GFP*

*Bx$^{Ms1096}$-GAL4*; *UAS-p35*/ CyO *Act GFP*

*Lz-GAL4*; *spag $^{k12101}$*/CyO

*UAS-PTEN-RNAi* (KK 101475); *UAS-Ras$^{V12}$*

*UAS-scrib-RNAi* (KK 105412); *UAS-Ras*$^{V12}$

*UAS-scrib-RNAi* (KK 105412); *UAS-p35*

*egr*$^{3}$/SM1; *VNO*/TM3 *Sb*

# 4 Results

## 4.1 The activation of PPO in Dipterans and Lepidopterans: Microbial elicitors versus danger signals

Phenoloxidase (PO) is a key enzyme of invertebrate immunity. Proteolytic activation of its zymogen prophenoloxidase (PPO) leads to the production of cytotoxic intermediates and results ultimately in visible melanization, which can hence serve as a read out for PO activity. To learn more about the activation of PPO I compared hemolymph and clot preparations from larvae of four insect species, two Lepidopterans (*Galleria mellonella* and *Pararge aegeria*) and two Dipterans (*Drosophila melanogaster* and *Anopheles gambiae*).

### 4.1.1 Melanization patterns in the clot of Dipteran and Lepidopteran larvae are different

To study PPO activation morphologically, larvae were bled and the forming clot captured and examined under the light microscope. Melanization became visible after approx. 5 min in all species compared. In *Drosophila* melanization was restricted to the surface of the hemolymph drop and localized in stripes or dots in a random pattern (Figure 11, C). In contrast, melanization in *Galleria* was systemic, i.e. homogenously distributed in the hemolymph (Figure 11, A).

To test if these two modes of PPO activation are also common in other insects, another Dipteran species, *Anopheles gambiae* and another Lepidopteran, *Pararge aegeria* were bled and melanization assessed as for *Drosophila* and *Galleria*. Interestingly melanization in both Lepidopteran species, *Galleria* and *Pararge* was systemic (Figure 11, A-B´´) while in the two Dipterans, *Drosophila* and *Anopheles* melanization remained localized and was restricted to parts of the clot (Figure 11, C-D´´). Possibly, inhibitory serpins are absent in the hemolymph of the two Lepidopterans. Another explanation might be that the targets of PO complexes differ in Lepidopterans and Dipterans. The fact that bleeds and clots of non-infected, healthy individuals from all four species melanized suggested that melanization could

be activated in the absence of microbial elicitors. In order to understand the cause of melanization I went on to characterize PPO activation on a molecular level.

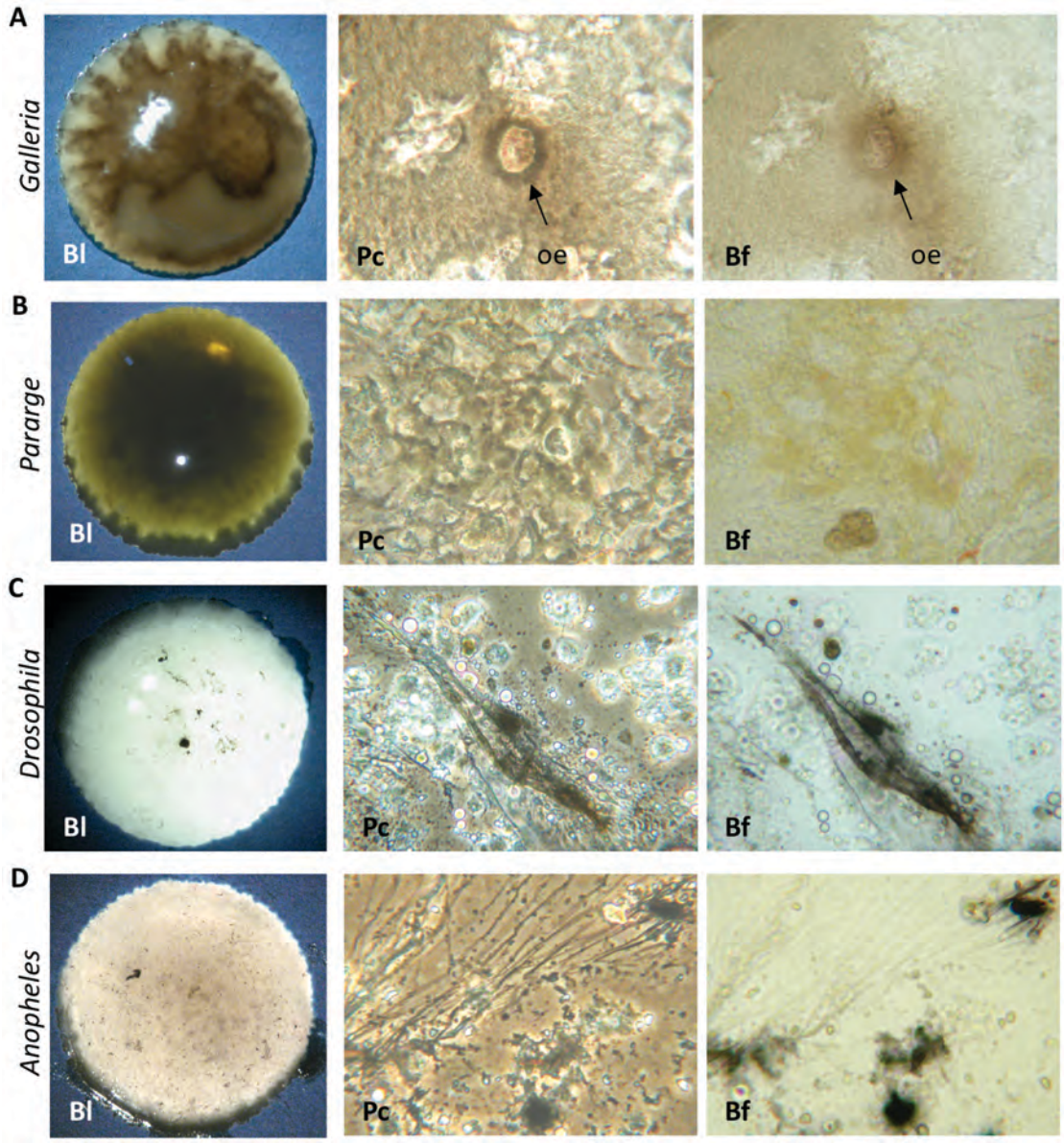


**Figure 11. Melanization patterns in the clot of Dipteran and Lepidopteran larvae differ.** (A) *Galleria* blood smears (Bl) blackened systemically in hanging drop preparations as did blood smears from *Pararge* larvae (B). In contrast, in *Drosophila* (C) and in *Anopheles* (D) melanization remained locally restricted. Phase contrast (Pc) and Bright field (Bf) images of clot preparations are shown: Melanization localized to clot folds in *Drosophila* and *Anopheles* while being diffuse in the two Lepidopterans. An oenocytoid (oe) is captured in the clot. Figure adapted from (Bidla, Hauling et al. 2009).

### 4.1.2 Injection of bacterial elicitors into the larval hemocoel provokes systemic melanization in *Galleria* but not in *Drosophila*

*Galleria* and *Drosophila* can easily be reared on artificial diet under controlled conditions and were hence chosen to further study PPO activation.

The bacterial elicitor peptidoglycan (PGN) was injected into the hemocoel of larvae from both species. 90 min post injection, *Galleria* exhibited systemic melanization throughout the hemocoel (Figure 12, A and A´) while in *Drosophila* melanization remained at the site of injection as expected from the melanization pattern observed in the clot (Figure 12, B and B´).

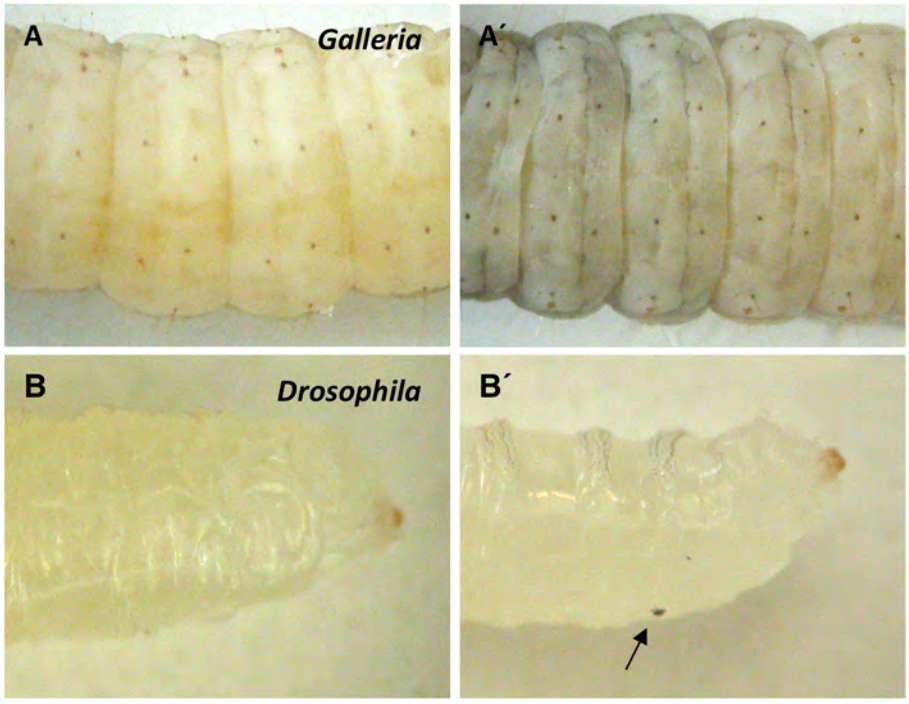


**Figure 12. Injection of a bacterial elicitor systemically activated PPO in *Galleria* but not in *Drosophila*.** (A) *Galleria* larva injected with bacterial peptidoglycan after 5 min. (A') 90 min post-injection systemic melanization was clearly visible. (B) The situation differed in *Drosophila*. 90 min post-injection larvae showed melanization at the injection site only (B'). Figure adapted from (Bidla, Hauling et al. 2009).

### 4.1.3 Endogenous danger signals activate *Drosophila* PPO after injury

It is well known from platelet activation in vertebrate blood clotting that inner-membrane lipids can serve as endogenous activators by providing a catalytic surface for clotting enzymes (Barry and FitzGerald 1999; Horstman and Ahn 1999; Morel, Morel et al. 2008). From previous data demonstrating that crystal cell activation in the clot involves cell lysis (Bidla, Dushay et al. 2007), I reasoned that PPO might be

activated in a similar way by fragments of ruptured crystal cells. Based on previous result by Gawa Bidla I confirmed that PO activity could be significantly superinduced by two inner-membrane phospholipids, phosphatidyserine (PS) and phosphatidylinositol (PI) while none of the outer-membrane phospholipids led to an increase in melanization (Figure 13, B). Activation of PPO was measured *in vitro* in hemolymph preparations as the change of optical density (OD) over time. Consistent with this data formation of melanotic bodies could also be triggered in larval hemolymph by genetically inducing apoptosis in a subset of hemocytes (Figure 13, D) resulting in exposure of inner-membrane lipids to immune factors present in the hemolymph. Here, a hemocyte-specific GAL4 driver (*Hemese GAL4*) was recombined with *tubulin* GAL80$^{ts}$ and crossed to a UAS-responder line carrying the apoptosis-inducer grim (Wing, Schwartz et al. 2001) to allow for time-specific induction of apoptosis in 3$^{rd}$ instar larvae that have a fully developed immune system (see 3.16 for details).

In contrast, in *Galleria* two bacterial elicitors superinduced activation of PPO. PGN and to a lesser extent lipopolysaccharide (LPS) led to a significant increase in melanization while PS and PI did not differ significantly from the control (Figure 13, C). Having demonstrated that melanization is triggered by endogenous factors in *Drosophila* hemolymph *in vitro* I went on to characterize PPO activation in the clot.

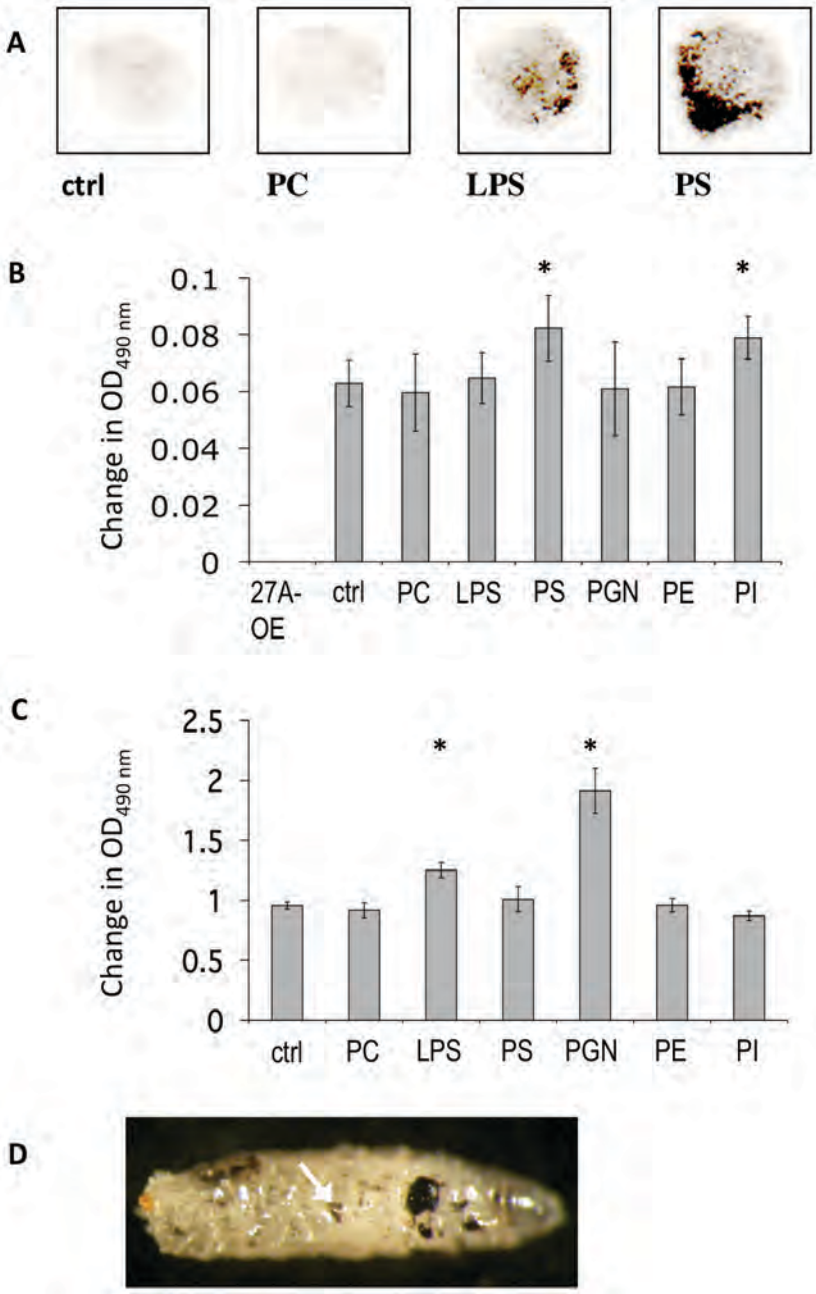


**Figure 13. Endogenous danger signals activate PPO in *Drosophila* but not in *Galleria*.** (A) Dot blots of hemolymph preparations blended with outer-membrane lipid phosphatidylcholine (PC), bacterial lipopolysaccharide (LPS) and inner-membrane lipid phosphatidylserine (PS), each dissolved in phospahate buffered saline (PBS). PS and to a lesser extent LPS superinduced melanization while PC and PBS only (ctrl) did not. (B and C) Photometric quantification of hemolymph melanization: In *Drosophila* (B) PS and phosphatidylinositol (PI) significantly superinduced melanization while ubiquitous expression of Spn27A in larvae completely abolished melanization of hemolymph preparations (27A-OE). The bacterial elicitors LPS and peptidoglycan (PGN) and the outer-membrane phospholipids PC and phosphatidylethanolamine (PE) did not increase melanization. Samples that differed significantly from the control are indicated by asteriks. P-values are: PS: 0.014: PI: 0.038: LPS: 0.0019: PGN: 0.00095. In *Galleria* (C) only the bacterial elicitors LPS and PBS superinduced melanization. Ctrl: sample with solvent (PBS) only. (D) In *Drosophila*, the formation of melanotic bodies (white arrow) in the hemolymph was induced *in vivo* by ectopically activating apoptosis in wandering stage larvae (*Hemese GAL4 tubGAL80$^{ts}$ > UAS-grim*). Figure adapted from (Bidla, Hauling et al. 2009).

### 4.1.4 Knockdown of *spn27a* leads to systemic melanization at the expense of clot melanization

In non-infected naive larvae, PO activity is limited to the clot matrix, where it contributes to clot polymerization and sequestration of invading microorganisms (Theopold, Schmidt et al. 2004; Bidla, Lindgren et al. 2005). Following infection in larvae and flies, the serine protease inhibitor (serpin) Spn27A (CG 11331), which has been shown to inhibit PPO activation in larvae *in vivo,* is transiently degraded thus enabling systemic activation of the enzyme. Spn27A loss-of-function mutants hence resemble infected larvae in terms of PO activity. As a consequence, PO activity in the hemolymph is higher than in unchallenged wt larvae (Ligoxygakis 2002; De Gregorio 2002). As expected, bleeds of *spn27A*$^{1}$ larvae showed systemic melanization similar to bleeds from *Galleria* and *Pararge* larvae (Figure 14, A). However, clot preparations from these mutants did not show any signs of melanization (Figure 14, B and C). This result was confirmed in another mutant that mimics infected larvae due to constitutively activated Toll signaling (*Tl*$^{8}$; data not shown). Bleeds from *Tl*$^{8}$ larvae contained melanotic aggregates as described previously (Braun, Hoffmann et al. 1998) but did not show any signs of systemic melanization or clot melanization. The absence of melanization can be due to actual inhibition of the PO cascade or caused by reduced substrate availability. By providing the PO substrate DOPA in clot preparations and dot blots of *spn27a*$^{1}$ mutants, melanization was rescued indicating that substrate availability was the limiting factor (Figure 14, D, E and inset in D). In contrast and in line with the role of Spn27A as a PO-inhibitor, melanization in hanging-drop preparations was completely inhibited in larvae with ubiquitous *spn27a* expression (Figure 13, B).

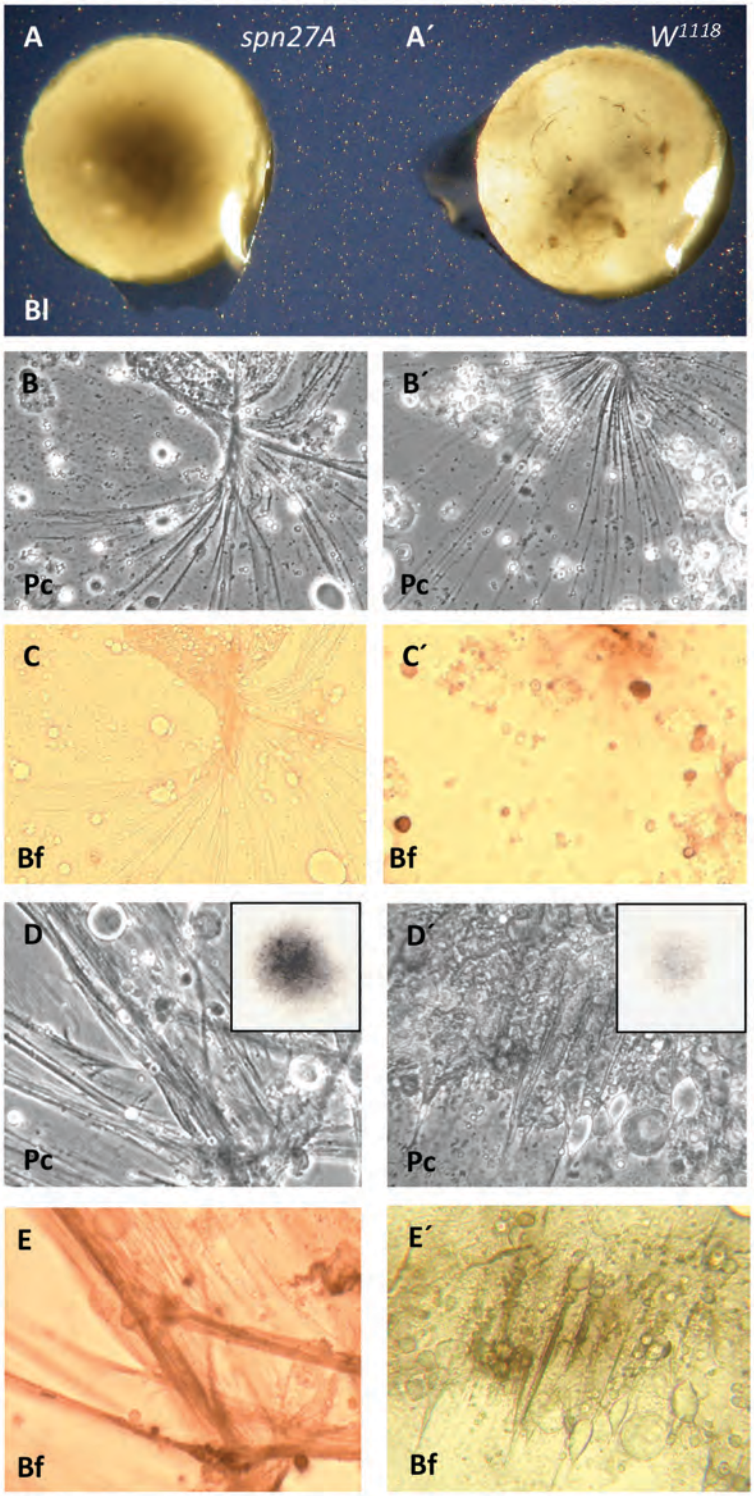


**Figure 14. *Spn27A* mutants lack clot melanization.** *Spn27A* mutants (A-E) were compared to $w^{1118}$ (wt, A'-E'). Blood smears from *spn27A* mutants melanized systemically (A) but did not show melanization in the clot (B and C) compared to wt (A'-C'). Shown are phase contrast (Pc) and bright field (Bf) images of a hanging drop preparation. Providing 3,4-dihydroxyphenylalanine (DOPA) in dot blots revealed increased PO activity in the hemolymph of *spn27A* larvae (inset in D) compared to wt (inset in D'). Adding DOPA rescued melanization in clot preparations from *spn27A* blood smears (D-E) to wt level (D'-E'). Figure adapted from (Bidla, Hauling et al. 2009).

### 4.1.5 Ectopic induction of apoptosis does not generally activate PPO

Having demonstrated that PS can superinduce melanization *in vitro* and that induction of apoptosis in a subset of hemocytes leads to formation of melanotic masses in the larval hemocoel I investigated if ectopically induced cell death in larvae generally provides danger signals to activate PPO *in vivo*. To test this hypothesis, I expressed pro-apoptotic genes and a cell toxin, ricin A chain, that has been used to ablate cells in *Drosophila* (Moffat, Gould et al. 1992; Sentry, Yang et al. 1993).

Contrary to my hypothesis, when inducing expression of pro-apoptotic genes (*grim*, *hid* and *reaper*) in imaginal discs, the salivary glands and the fat body, melanization was not observed (the results of ectopic induction of cell death are summarized in Table 1). Unlike hemocytes, larval tissue including the above mentioned organs is lined by an extracellular layer, the basement membrane, which might serve as a protective barrier that prevents access of patrolling immune cells. In agreement with this hypothesis, melanotic bodies formed when apoptosis was induced in a subpopulation of hemocytes. Dying hemocytes would readily be accessible for components of the PPO cascade. The fact that melanotic masses, which exceeded the size of single hemocytes, mostly floated in the hemolymph suggests that hemocytes formed aggregates, possibly similar to what is observed in class 2 melanotic “tumor” mutants (see paragraph 1.3 and (Watson, Johnson et al. 1991)). In conclusion, the data indicates that apoptosis is not generally immunogenic but can trigger the PPO cascade under circumstances not yet fully understood.

**Table 1. PPO activation by ectopic expression of cell death inducers.** The induction of transgenes has been carried out at 25° and 29°C unless noted otherwise. (--): no melanisation; (o): small melanotic masses; (+): large melanotic masses (additional observations are stated in brackets)

| **Expression profile of GAL 4 driver (additional information in brackets)** | **Melanization (additional observations in brackets)** |
|---|---|
| **GAL4 driver > UAS-grim** | |
| imaginal discs, brain (*c701b-GAL4*) | -- (lethal) |
| eye disc (*GmR-GAL4*) | -- (reduced eyes in adults) |
| wing disc and salivary glands (*BxMS1096-GAL4*) | -- (extended larval period, reduced wings in adults) |
| salivary glands (*Sgs3-GAL4*) | -- |
| late 3rd instar fat body (*Lsp2-GAL4*) | -- (larvae more transparent) |
| fat body and salivary glands (*5253 GAL4*) | -- (lethal in larval stage, larvae are smaller than wt) |
| hemocytes (*he-GAL4*, *tub-GAL80ts* as described in 3.16) | + (melanotic bodies in hemolymph) |
| Hemocytes (*he-GAL4*) | o (melanotic bodies in hemolymph) |
| ubiquitously in all tissues (Heat shock GAL4 (heat shock at 37°C for 1h, then transfer to 25°C) | **+** (lethal, melanization variable, throughout the animal) |
| **GAL4 driver > UAS-hid** | |
| imaginal discs, brain (c701b-GAL4) | lethal |
| late 3rd instar fat body (*Lsp2-GAL4*) | -- |
| fat body and salivary glands (*5253 GAL4*) | -- (lethal in larval stage) |
| eye disc (*GmR-GAL4*) | -- (lethal in pupal stage) |
| **GAL4 driver > UAS-reaper** | |
| late 3rd instar fat body (*Lsp2-GAL4)* | -- |
| eye disc (*GmR-GAL4*) | -- (lethal in pupal stage) |
| **GAL4 driver > UAS-ricin/ CyO GFP** | |
| eye disc (*GmR-GAL4*) | -- (reduced eyes in adults) |
| wing disc and salivary glands (*BxMS1096-GAL4*) | -- (lethal) |
| late 3rd instar fat body (*Lsp2-GAL4*) | -- |

## 4.2 The activation of the innate immune system by altered self in *Drosophila* larvae

Mutants in which immune reactions are initiated against aberrant tissue with apparently altered properties have previously been described in *Drosophila* (Watson, Johnson et al. 1991; Minakhina and Steward 2006). I studied the immune response against aberrant, tumor-like tissue and compared this response to an autoimmune mutant, *spag*$^{k12101}$, which is characterized by auto-encapsulation of non-

hematopoietic larval tissue, including the fat body and imaginal discs (Minakhina and Steward 2006).

### 4.2.1 Induction of tumor-like altered self with the GAL 4 system

Homozygous loss-of-function mutations in a number of tumor suppressor genes have been shown to cause tumorous overgrowth in various tissues including imaginal discs, brain and hematopoietic tissue in larvae (Gateff 1978). Furthermore, aberrant overgrowth, which inhibited developmental degradation during pupal stage has also been induced in larval salivary glands by expressing $Ras^{V12}$, a dominant-active form of the proto-oncogene Ras1 (Berry and Baehrecke 2007).

I reasoned that by knocking down described and characterized tumor suppressor genes it should be possible to recapitulate the phenotype described for the respective loss-of-function mutants. When driving RNAi-knockdown-constructs targeting the tumor suppressor *lethal giant larvae* (*l(2)gl*; see section 1.7.1) under the control of ubiquitously expressed GAL4 driver lines (*Actin 5C GAL4* and *daughterless* GAL4) larvae overgrew compared to wt larvae ($w^{1118}$) and died between late wandering stage and pupal stage (see Figure 15). Essentially, ubiquitous *l(2)gl* knockdown phenocopied homozygous mutant larvae of the respective gene (Gateff and Schneiderman 1969).


**Figure 15. Larval overgrowth as a result of ubiquitous tumor suppressor knockdown.** *Lethal giant larvae* (*l(2)gl*) was knocked down ubiquitously by expressing a *UAS-l(2)gl$^{RNAi}$* construct (VDRC line 51249/GD) with *daughterless-GAL4* (*l(2)gl$^{RNAi}$*) resulting in larval overgrowth compared to wildtype (wt). Depicted are pupae. Scale bar: 0.5 mm

Using the same GAL4-drivers pupal lethality was also observed when the tumor suppressors *fat* (see paragraph 1.7.2 and (Watson, Justice et al. 1994)) and *bunched* (Gluderer, Oldham et al. 2008; Wu, Yamada-Mabuchi et al. 2008) were knocked down. I selected those RNAi lines that caused an overgrowth phenotype and crossed them to

GAL4 driver lines, which are not expressed in immune tissue, i.e. imaginal discs and salivary glands. The goal was to find a GAL4-driver, that when crossed to tumor-suppressor$^{RNAi}$ responder-lines or oncogene over-expression responder-lines causes a measurable phenotype in the offspring that can be distinguished from parental controls and allows for complete larval development. Of all lines tested, only *Beadex$^{ms1096}$–GAL4* (*Bx-GAL4*), which is expressed in the dorsal part of wing imaginal discs and in the larval salivary glands, matched these requirements. The knockdown of *l(2)gl* with this driver allowed for complete devlopment to adult stage. Eclosing flies had partially blackened wings that did not fully unfold (Figure 16, A). *L(2)gl*$^{RNAi}$ wing imaginal discs from 3$^{rd}$ instar larvae exhibited overgrowth compared to the wildtype (Figure 16, B).

**Table 2. GAL4 driver screen with tumor-suppressor$^{RNAi}$ and Ras$^{V12}$ responder lines.** GAL4 drivers as specified in the table have been crossed to specified responder lines and crosses were kept at 29°C. Effects on the offspring were assed by inspection of larval development and adults. The observed phenotype is stated in the table.

| **Expression pattern of GAL 4 driver (all drivers are also expressed in larval salivary glands)** | **Phenotype (targeted genes in brackets)** |
|---|---|
| Ubiquitous (*daughterless-GAL4*) | Lethal (*l(2)gl$^{RNAi}$*, *fat$^{RNAi}$*, *TSC-22$^{RNAi}$*), no obvious defects (*p53$^{RNAi}$*, *l(3)mbt$^{RNAi}$*, *dl1$^{RNAi}$*) |
| Ubiquitous (*Actin 5C*-GAL4) | Lethal (*l(2)gl$^{RNAi}$*, *fat$^{RNAi}$*, *TSC-22$^{RNAi}$*), no obvious defects (*p53$^{RNAi}$*, *l(3)mbt$^{RNAi}$*, *dl1$^{RNAi}$*) |
| Eye disc (*GMR-GAL4*) | Lethal (Ras$^{V12}$), no defects (*l(2)gl$^{RNAi}$*) |
| Wing disc (*Bx$^{MS1096}$-GAL4*) | Aberrant wings (*l(2)gl$^{RNAi}$*), lethal (Ras$^{V12}$) |
| Imaginal discs (*32B-GAL4* and *71B-GAL4*) | Lethal (Ras$^{V12}$) |
| 3$^{rd}$ instar imaginal discs (*T80-GAL4* and *c701b-GAL4*) | No defects (Ras$^{V12}$, *l(2)gl$^{RNAi}$, warts$^{RNAi}$*) |
| Antennal imaginal discs (*OK384-GAL4*) | No defects (Ras$^{V12}$) |
| Late 3$^{rd}$ instar fat body (*Lsp2-GAL4*) | No defects (Ras$^{V12}$) |
| Fat body (*5253-GAL4*) | Lethal in pupal stage (Ras$^{V12}$) |
| Fat body (*5267-GAL4*) | Lethal in pupal stage (Ras$^{V12}$) |
| Fat body (*Ppl-GAL4*) | Larval lethal, small larvae (Ras$^{V12}$) |

Crossing *Bx-GAL4* to flies carrying *UAS-RNAi* responders that target genes of the Hippo pathway (*fat* and *warts*; see section 1.7.2) caused development of wings that were enlarged compared to their wt counterparts (Figure 16, A). Wing imaginal discs

from 3rd instar larvae appeared similar to wt, which is in line with the milder wing phenotype compared to *l(2)gl* RNAi knockdown (Figure 16, B).

Expressing $Ras^{V12}$ resulted in the severest aberration leading to clearly overgrown and disorganized wing discs and salivary glands in larvae, eventually resulting in pupal lethality. Lethality was apparently due to $Ras^{V12}$ expressing wing discs since larvae that expressed the oncogene in salivary glands alone (*Sgs3-GAL4*) developed into flies that did not show any morphological defects (not shown). The results of crosses between *Bx-GAL4* and tumor-suppressor/ oncogene responder lines are summarized in Table 3.

**Table 3. Mini screen with *Bx-GAL4*: the driver was crossed to *UAS-tumor-suppressor$^{RNAi}$* and *UAS-Ras$^{V12}$* responders.** The crosses were kept at 29°C except for crosses with *UAS-Ras$^{V12}$*, which were performed at 29°C and 25°C. (-): like wt; (o): deformed, overgrown wings; (+): deformed, partially blackened wings; (++): melanotic spots in pupae, associated with pupal lethality

| **Tumor suppressor$^{RNAi}$/ oncogene responder** | **Phenotype** |
|---|---|
| *l(2)gl* | + (three lines tested) |
| *scrib* | o |
| *warts* | o |
| *fat* | o |
| *hippo* | - |
| *disc large* | - |
| *P53* | - (two lines tested) |
| *PTEN* | o |
| *bunched* (*TSC-22*) | - (two lines tested) |
| *Ras$^{V12}$* | ++ (lethal at 25°C and 29°C) |
| *Ras$^{V12}$* (expression partially suppressed with GAL80$^{ts}$) | + at 25°C (lethal at 29°C) |

Strikingly, pupae that expressed $Ras^{V12}$ under *Bx-GAL4* exhibited blackened areas that co-localized with developing wings (Figure 16, C). Lethality was apparently dependent on the dosage of the *Ras$^{V12}$* transcript since attenuating *Ras$^{V12}$* expression with *tubulin GAL80$^{ts}$* (TARGET system, described in (McGuire, Le et al. 2003) and paragraph 3.17) rescued pupae and allowed them to develop into adults. Eclosing flies had aberrant wings similar to *l(2)gl* RNAi-knockdown flies when the cross was kept below the permissive temperature of the TARGET system at 25 ˚C (Figure 16, A and C). At 18 ˚C wings developed like in wildtype flies indicating that *Ras$^{V12}$* expression was further reduced to levels that did not disturb wing development (Figure 16, C).

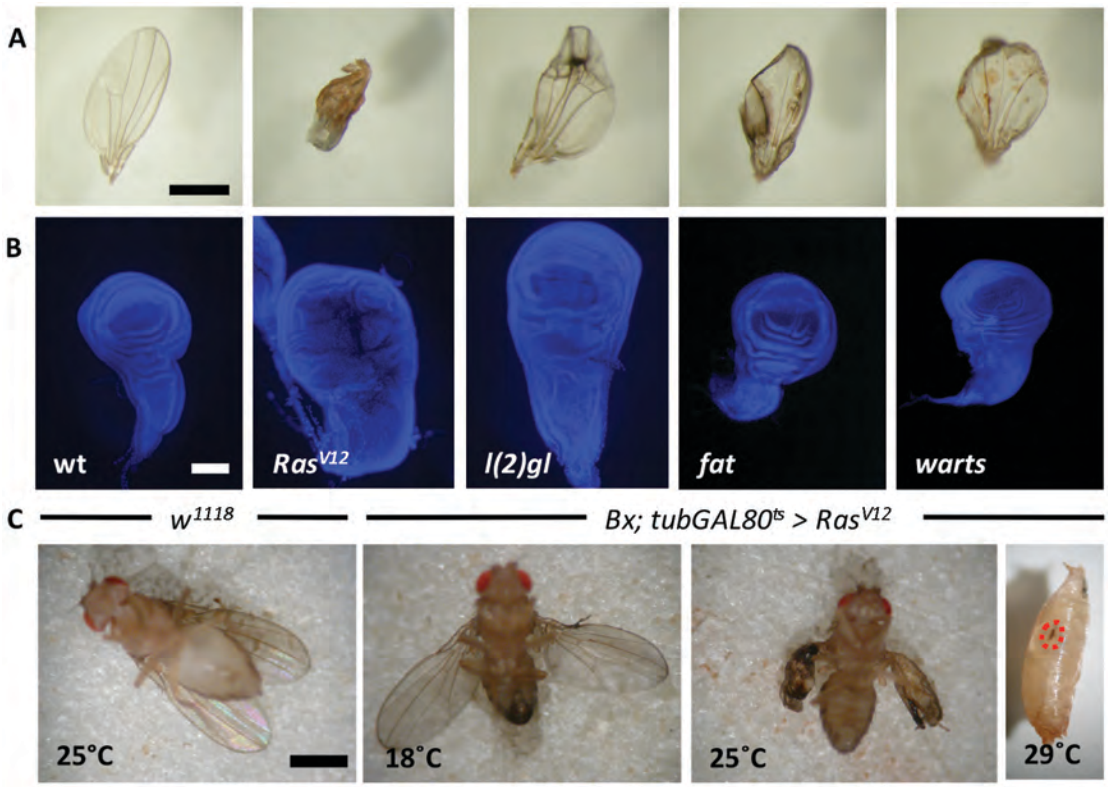


**Figure 16. Knockdown of tumor suppressors and oncogene expression induced aberrant tissue growth in larvae and melanization in pupae and adults.** (A) RNAi knockdown of tumor suppressors (*l(2)gl*, *fat* and *warts*) or expression of oncogenic Ras$^{V12}$ in wing imaginal discs and salivary glands with *Bx-GAL4* led to formation of aberrant wings compared to wt. Note that Ras$^{V12}$ expression has been attenuated by coexpressing the GAL4 inhibitor GAL80$^{ts}$ (*Bx GAL4;tub GAL80$^{ts}$ > UAS-Ras$^{V12}$* – short: *Bx;tubGAL80$^{ts}$*>Ras$^{V12}$) since full induction of Ras$^{V12}$ resulted in pupal lethality characterized by melanotic spots (enclosed by dotted line in C, 29˚C). (B) Using the same GAL4 driver a loss of structural organization compared to wt was observed in wing imaginal discs when Ras$^{V12}$ or an *l(2)gl RNAi* construct were expressed. No obvious defects in wing discs were observed when *fat* and *warts* were knocked down. (C) The Ras$^{V12}$ wing phenotype could be modified by attenuating Ras$^{V12}$ expression with the temperature-sensitive GAL4 inhibitor GAL80$^{ts}$ (see paragraph 3.17 for details): At 18˚C the wings were slightly enlarged compared to wt (*w$^{1118}$*), while increased expression at 25˚C resulted in a melanotic wing phenotype. Complete release of GAL4 repression at 29°C led to pupal lethality with characteristic melanotic spots (dotted red line).

Since the strongest aberrant phenotype was observed when Ras$^{V12}$ was expressed I picked this candidate for the induction of aberrant self in the following study.

### 4.2.2 Characterization of Ras$^{V12}$ aberrant tissue

To follow the development of Ras$^{V12}$ wing discs and salivary glands I co-expressed *UAS-Ras$^{V12}$* and *UAS-GFP* thus labeling the aberrant tissues. Respective larvae that co-expressed the transgenes under *Bx-GAL4* or under a salivary gland specific driver (*Sgs3-GAL4*, not shown) contained GFP-positive masses in their hemolymph, which had apparently dissociated from their origin (Figure 17, A). One feature that distinguishes malignant tumors from normal tissue is their invasiveness. Tumor cells

that invade their environment have to breach a barrier, the basement membrane that surrounds them.

Often they actively degrade the basement membrane (or extracellular matrix) by expressing proteolytic enzymes, therefore named matrix metalloproteases (MMPs). *Drosophila* possesses two genes that encode MMPs – MMP1 and MMP2 (Llano, Adam et al. 2002). MMP1 has also been shown to facilitate invasiveness of imaginal disc tumors in *Drosophila* previously (Uhlirova and Bohmann 2006). These tumors originated from single clones that were deficient for a gene of the apicobasal polarity module and that expressed activated Ras. In order to test whether $Ras^{V12}$ expression alone suffices to induce MMP1 expression in the aberrant tissue I performed immunohistochemistry to detect the enzyme using a commercially available anti-MMP1 antibody. Indeed a large fraction of $Ras^{V12}$ salivary gland cells expressed MMP1 while no expression of the protease was detected in wt salivary glands (Figure 17, B and C). Likewise knockdown of *l(2)gl* in salivary glands did not induce MMP1 expression (Figure 18, B). In line with previous results, $Ras^{V12}$ expression alone did not trigger MMP1 production in wing imaginal discs (Figure 19, D).

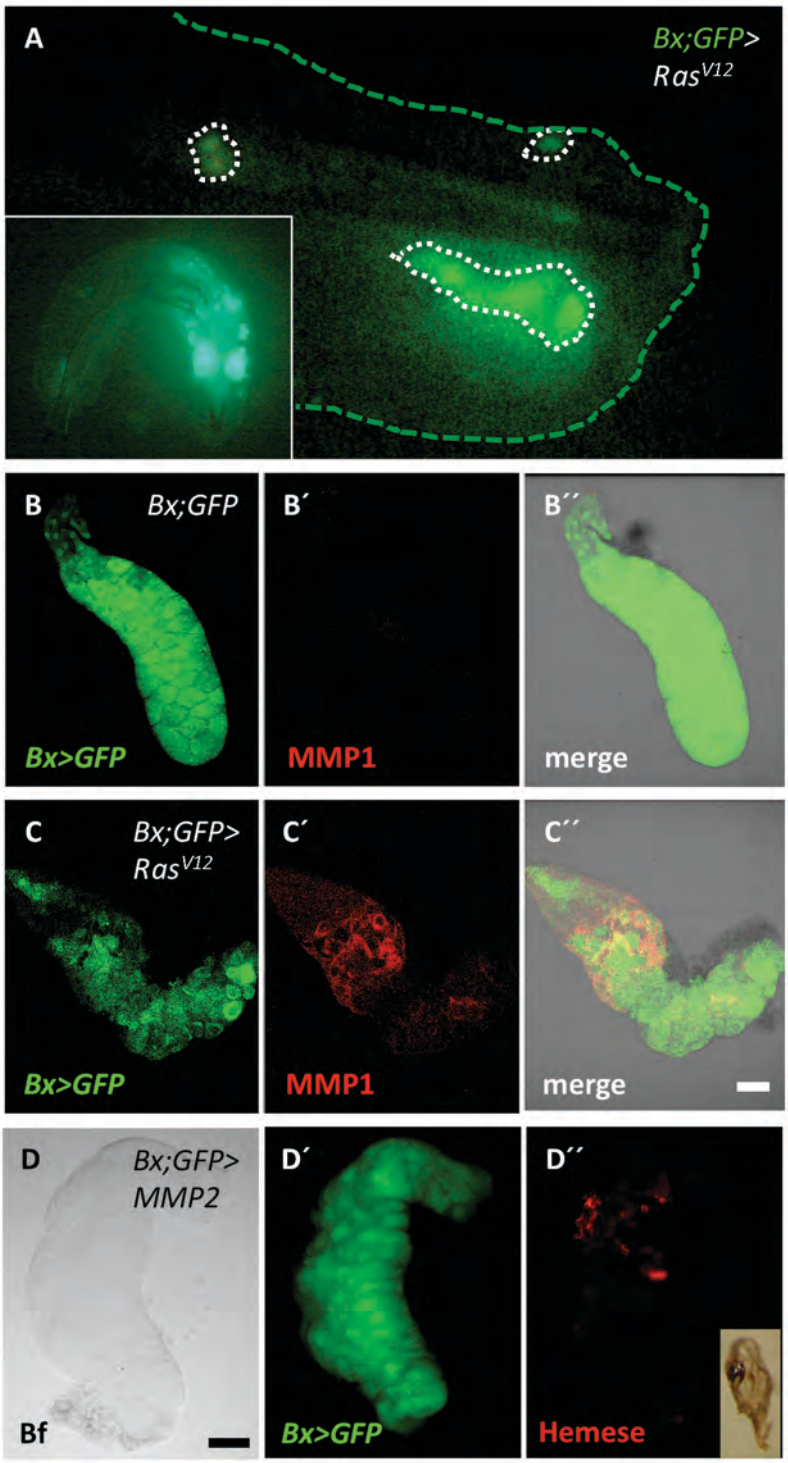


**Figure 17. Ras$^{V12}$ salivary glands express MMP1 and putatively invade the surrounding hemocoel.** (A) Stereomicroscopical image of the posterior part of a live larva (green dashed line), expressing Ras$^{V12}$ and GFP in wing discs and salivary glands (*Bx GAL4*; *UAS-GFP* > *UAS-Ras$^{V12}$* – short: *Bx*;*GFP*>Ras$^{V12}$) The inset depicts the whole larva. GFP-positive bodies (white dotted line) were found throughout the hemocoel. (B and C´´) Single projections of Z-stacks of confocal sections: (B´) Wildtype salivary glands did not express MMP1 in contrast to Ras$^{V12}$ glands (C´). The glands have been labeled by coexpression of GFP (*Bx*>GFP in B and C). (D – D´´) Expression of MMP2 in wing imaginal discs and salivary glands with *Bx-GAL4* induced hemocyte adherance and melanization. (D) Bright field image (Bf) of an MMP2-expressing salivary gland. The gland has been labeled by coexpression of GFP (D´). Hemocytes adhered to the gland (Hemese in D´´). MMP2 was also expressed in wing discs and resulted in melanization of the wings (inset in D´´). scalebar: 100 µm.

Breakdown products of the extracellular matrix have been proposed to act as danger signals that trigger immune responses in insects (Altincicek, Linder et al. 2007; Altincicek and Vilcinskas 2008) and ectopic MMP expression in imaginal discs has been shown to attract hemocytes to the respective tissue in *Drosophila* (Pastor-Pareja, Wu et al. 2008).

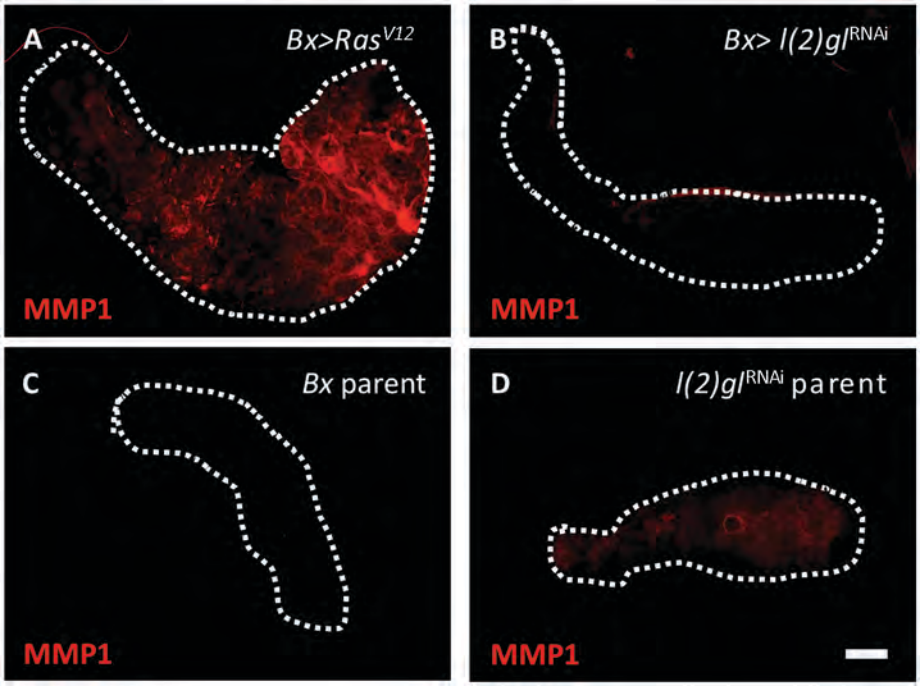


**Figure 18. RNAi knockdown of the tumor suppressor *l(2)gl* did not induce MMP1 expression.** Shown are epifluorescence images of salivary glands (encircled by dotted line). (A) MMP1 (red signal) was detected in the positive control (Ras$^{V12}$ salivary gland) but absent when *l(2)gl* was knocked down (B) No MMP1 was detected in the parental controls (C and D). *Bx-GAL4* was used to drive expression of the UAS transgenes. Scale bar: 100 µm.

In line with the latter I found that expressing MMP2 (which can substitute MMP1 to disrupt the basement membrane (Pastor-Pareja, Wu et al. 2008) under *Bx-GAL4* resulted in a few hemocytes attaching to the salivary glands. Furthermore, the wings of eclosing flies exhibited a melanotic phenotype that was reminiscent of wing melanization observed in *Bx*;*tubGAL80$^{ts}$*>Ras$^{V12}$ flies.

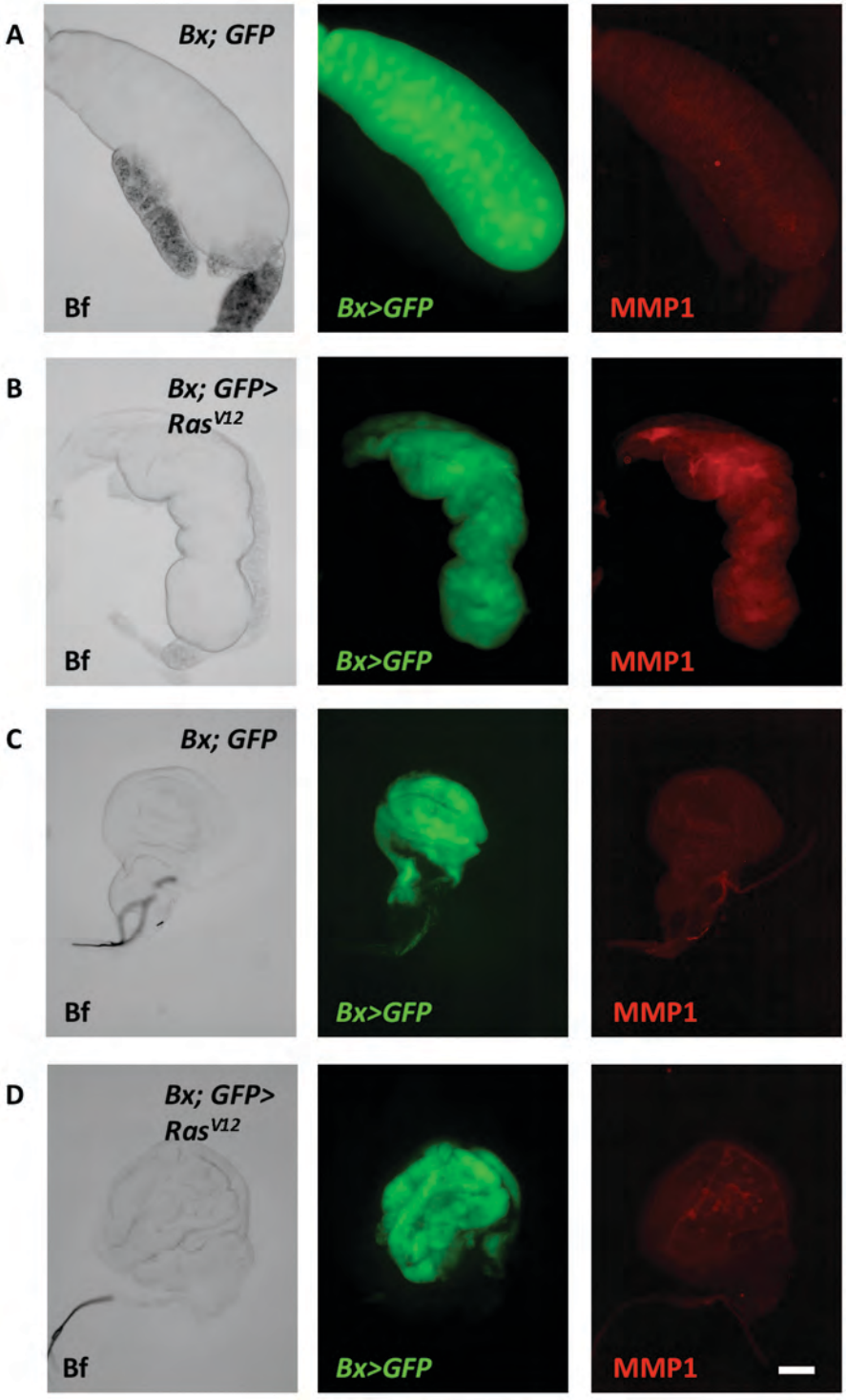


**Figure 19. MMP1 is not expressed in Ras$^{V12}$ wing imaginal discs.** MMP1 was detected in a Ras$^{V12}$ salivary gland (B) but not in a Ras$^{V12}$ wing disc (D). MMP1 was also absent in salivary gland and wing disc of the parental control (*Bx*; GFP). The organs were labeled by GFP coexpression (*Bx GAL4* > *UAS-GFP* – short: *Bx*>GFP). Bright field images are depicted in the left column (Bf). The remaining columns show epifluorescence images. Scale bar: 100 µm.

### 4.2.3 The cellular immune response against Ras$^{V12}$ aberrant salivary gland is encapsulation

I decided to investigate hemocyte attachment on Ras$^{V12}$ salivary glands and wing discs since Ras$^{V12}$ salivary glands (but not wing imaginal discs) express MMP and MMP expression under *Bx-GAL4* results in adherence of hemocytes to salivary glands. Aberrant wing discs and salivary glands were tested for hemocytes with antibodies for the transmembrane protein Hemese, which is expressed by all hemocyte classes (Kurucz, Zettervall et al. 2003). I found hemocytes on Ras$^{V12}$ expressing salivary glands but not on wt glands (Figure 20, B-C´´). Hemocytes were also detected on wing imaginal discs. However, variable numbers of hemocytes could be observed on both Ras$^{V12}$-expressing and control imaginal discs (Figure 20, A and A´). To be certain that hemocytes attach to wing imaginal discs *in vivo*, I dissected and immediately imaged these organs from larvae that constitutively expressed GFP in hemocytes under the *tep1* promoter. In this way labeled hemocytes were detected on wt discs thus confirming the immunohistochemistry result (see section 3.11.1).

The reason for the presence of hemocytes on wt wing imaginal discs is unknown but is in line with results from others (Pastor-Pareja, Wu et al. 2008). From microscopical inspection, I could not detect a difference in number of adhering hemocytes on Ras$^{V12}$ and control wing discs.

Hence, contrary to salivary glands, Ras$^{V12}$ expression in wing discs did not appear to attract hemocytes (tested with the same result were also *l(2)gl*$^{RNAi}$, *fat*$^{RNAi}$, *warts*$^{RNAi}$). The results supports the idea that MMP mediated breakdown of the extracellular matrix must occur to allow for hemocyte attachment since MMP expression was not observed in Ras$^{V12}$ wing discs.

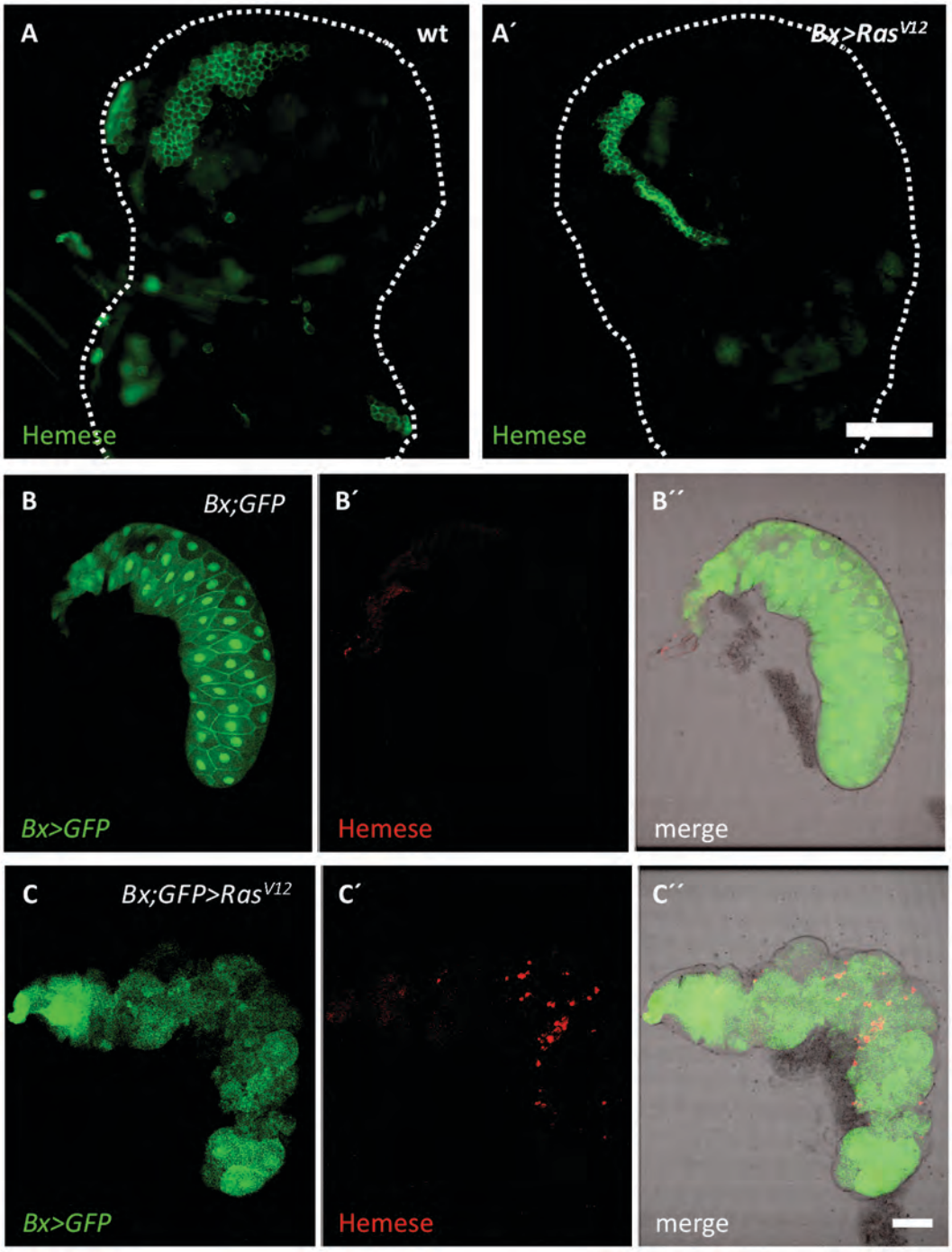


**Figure 20. Hemocytes adhere to wing imaginal discs and Ras$^{V12}$ salivary glands.** Epifluorescence images (A-A´) and single projections of Z-stacks of confocal sections (B-C) are shown: Hemese-positive hemocytes (Hemese, green color) adhered to both wt (A) and Ras$^{V12}$ (A´) wing imaginal discs (encircled by dotted line); scale bar: 50 µm. In contrast, no hemocytes were detected on wt (*Bx*>GFP) salivary glands (B´). Ras$^{V12}$ expression in salivary glands triggered hemocyte infiltration (C´). Salivary glands were labeled by coexpression of GFP (*Bx GAL4* > *UAS-GFP* – short: *Bx*>GFP in B and C). merge (B´´and C´´): merged views of the Z-stack projections and of the respective bright field images; scale bar: 100 µm.

The pattern of hemocyte adherance varied between salivary glands ranging from a few individual hemocytes to pronounced hemocytic patches. Hemocytes were

observed on all $Ras^{V12}$ expressing salivary glands retrieved from wandering stage 3rd instar larvae reared at 29°C (*n*= 12). On some salivary glands hemocytes formed massive layers on the tissue and spread around individual cells of the gland. This could also be observed when $Ras^{V12}$ was expressed in the fat body (5253>$Ras^{V12}$ and 5267>$Ras^{V12}$). In rare cases $Ras^{V12}$ expressing salivary glands and fat body melanized partially.

In *Drosophila,* melanization is induced upon wounding and PO-activity is also the final step of the encapsulation process, which is initiated against larger foreign objects (Williams 2007) and against aberrant self in certain autoimmune mutants such as $spag^{k12101}$ (Minakhina and Steward 2006).

Having detected pronounced hemocyte infiltration and melanization on $Ras^{V12}$ salivary glands and fat body I reasoned that the response against such tumor-like tissue might be encapsulation and hence lamellocytes, which are exclusively involved in this process, can therefore be used as a marker for encapsulation. Indeed, I detected this celltype on some $Ras^{V12}$ salivary glands (Figure 21; E and E´) Lamellocytes were also found on $Ras^{V12}$ fat body but not on $Ras^{V12}$ expressing wing discs (not shown). I used the L2-antibody that is specific for mature lamellocytes, which participate in encapsulation of parasitoid eggs (Honti, Csordas et al. 2010). Melanization of hemocyte-infiltrated tissue occurred rarely, similar to the autoimmune response in $spag^{k12101}$ larvae. Here, hemocytic layers including lamellocytes (i.e. capsules) formed on the fat body. Such capsules were melanized in some but not in all cases (Figure 21; D – D´´ and F, F´). In all specimens studied lamellocyte attachment correlated with other hemocytes, which were detected by their smaller nuclei compared to salivary gland or fat body cell nuclei (see Figure 7). This data suggested that lamellocytes require signals from other hemocytes for attachment. The respective hemocyte-derived signals remain to be elucidated.

In both $Ras^{V12}$ expressing fat body and salivary glands and tissue from $spag^{k12101}$ mutants encapsulation did not ensue systemically or evenly distributed. Instead a continuum of encapsulation steps was observed resulting in larvae with both melanotic and non-melanotic capsules (Figure 21).

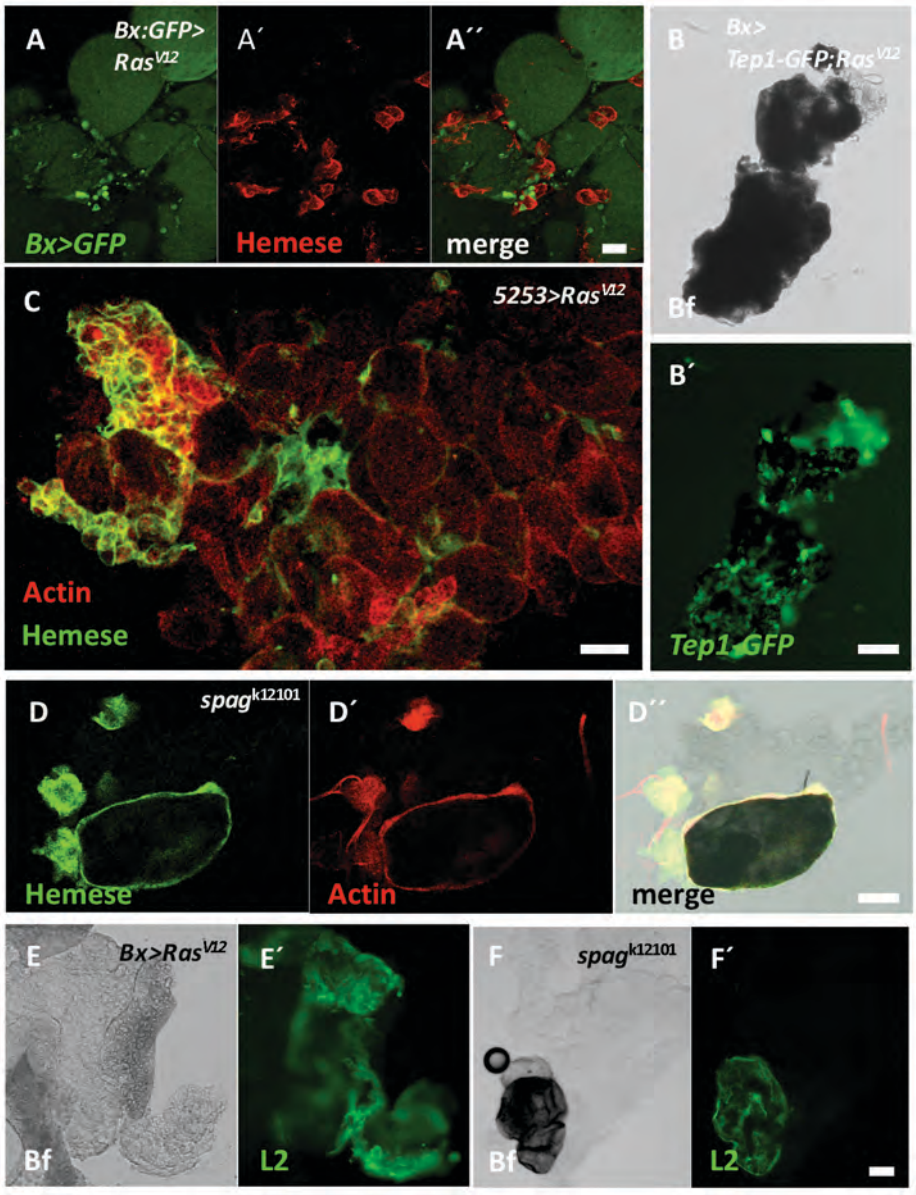


**Figure 21. Encapsulation is the response against Ras$^{V12}$ aberrant salivary gland and fat body.** Single projections of Z-stacks of confocal sections (A and C) and epifluorescence images (B, D-F) are shown. (A-A´´) section of a GFP-labeled Ras$^{V12}$ salivary gland with adhering hemocytes (Hemese, red); individual hemocytes spread around a salivary gland cell. (B-B'): a melanized Ras$^{V12}$ salivary gland. This phenotype was associated with GFP-labeled hemocytes *Tep1 GFP*). (C) Hemocyte-infiltration was also observed in Ras$^{V12}$ expressing fat body: hemocytes were labeled with anti-Hemese staining (Hemese, green). Actin was stained with TRITC-conjugated Phalloidin (Actin, red). (E) Encapsulation in *spag$^{k12101}$* larvae visualized by anti-Hemese labeling (Hemese, green). Melanotic and non-melanotic capsules were observed (merge). Lamellocytes were involved in encapsulation in both Ras$^{V12}$ salivary glands and *spag$^{k12101}$* larvae. Bright field (Bf) images of a Ras$^{V12}$ salivary gland (E) and fat body in *spag$^{k12101}$* (F). Mature L2-antigen expressing lamellocytes were detected by anti-L2 staining (L2, green) in E´ and F´. Scale bar in B-B´´: 20 µm. Scale bars in other images: 100 µm.

Crystal cells have been reported to be required for encapsulation-associated melanization (Rizki and Rizki 1990) and in general the function of crystal cells in immunity other than wound healing and coagulation is poorly understood. I hence investigated if crystal cells were present on aberrant salivary glands. Using PPO

antibody (seemingly intact) crystal cells were detected on Ras$^{V12}$ salivary glands but not on wt glands. These cells seemed to be non-activated since crystals were intact. Like lamellocytes, crystal cells were always found in conjunction with other hemocyte classes; no more than 7 crystal cells per salivary gland were detected (*n*= 10 glands). None of the glands were melanized. Crystal cell adherence hence seemed to precede PPO-release and activation of the PPO cascade.

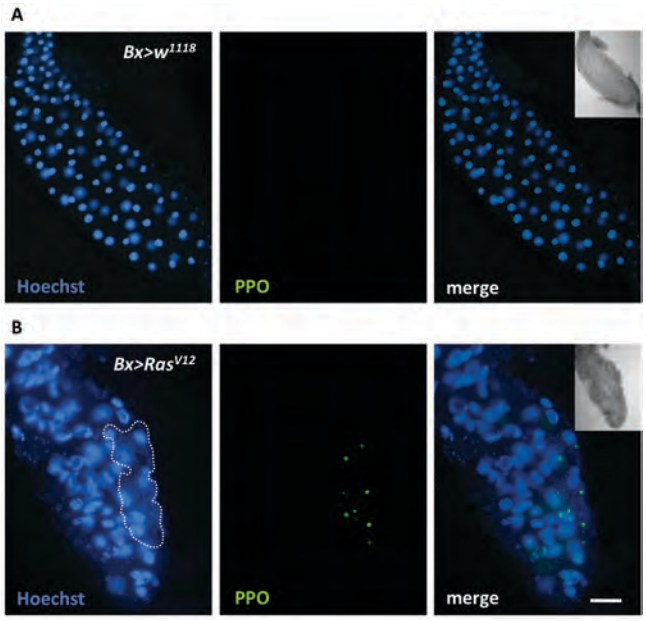


**Figure 22. Crystal cells adhere to Ras$^{V12}$ salivary glands.** Crystal cell specific mouse PPO antibody was detected with FITC-conjugated goat-anti-mouse antibody (PPO). Nuclear DNA was stained with Hoechst. (B) Crystal cells adhere in conjunction with other hemocytes (small nuclei enclosed by dashed line) onto a Ras$^{V12}$ salivary gland (*Bx GAL4 > UAS-Ras$^{V12}$* – short: *Bx>Ras$^{V12}$*). (A) No crystal cells were detected on wt (*Bx>w$^{1118}$*) salivary glands. Shown are epifluorescence images and merged views (merge). The insets in (merge) are bright field images of the respective glands. Scale bar: 100 µm.

In conclusion, Ras$^{V12}$ aberrant salivary glands and fat body triggered plasmatocyte infiltration that ultimately resulted in the recruitment of lamellocytes and crystal cells leading to encapsulation of self, which was occasionally accompanied by melanin deposition.

### 4.2.4 Encapsulation depends on Ras$^{V12}$ dosage

The encapsulation response was dependent on the strength of Ras$^{V12}$ expression. Ras$^{V12}$ was expressed under *Bx-GAL4*, which in turn was partially inhibited by *tubulin-GAL80$^{ts}$* (see paragraph 3.17). At 25 °C practically no lamellocytes adhered to the

glands since GAL80$^{ts}$ attenuated GAL4 activity, while release of GAL4 inhibition at 29˚C lead to an increase of lamellocyte adherence (Figure 23, A):

Approximately 10 % percent of Ras$^{V12}$ salivary glands were covered by patches of lamellocytes. More frequently salivary glands were infiltrated by hemocytes as revealed by their small nuclei compared to salivary gland cells but the majority of these cells did not stain with lamellocyte-specific L2 antibody. Instead either small clusters or individual L2-positive cells or no lamellocytes at all were detected. Lamellocyte numbers did not positively correlate with Ras$^{V12}$ expressivity. To my surprise, lamellocytes were not more abundant in circulation in larvae with Ras$^{V12}$ aberrant tissue at 29 ˚C compared to larvae with reduced Ras$^{V12}$-expression at lower temperatures. In fact, the opposite seemed to be the case, i.e. lamellocytes were more frequent in circulation in the parental line and larvae with low Ras$^{V12}$ expression compared to larvae with maximum induction of the oncogene (see Figure 23; B). Whether lamellocytes that were absent in circulation in a large percentage of larvae adhered to aberrant tissue or whether Ras$^{V12}$ induction in wing imaginal discs and salivary glands inhibits lamellocyte proliferation *in trans* remains to be elucidated.

The same Ras$^{V12}$ responder line has previously been used in a study of signaling pathways involved in encapsulation (Zettervall, Anderl et al. 2004) but uninfected larvae were not reported to contain lamellocytes. My finding was hence unexpected and I decided to investigate the presence of this celltype in other fly strains. 14 out of 15 lines contained lamellocytes in circulation, six of those lines contained considerable numbers of lamellocytes, i.e. more than 10 lamellocytes per blood smear. Here it has to be kept in mind that lamellocytes might not adhere to surfaces equally well than plasmatocytes do. The observed cell numbers might hence be underestimating the actual value. In conclusion, the screen suggested that lamellocytes differentiate readily in response to currently unknown stimuli (Table 4). Crucially, although present in circulation in the parental line, lamellocytes did not adhere to salivary glands (not shown).

**Table 4. Screen for lamellocyte proliferation in fly strains.** The blood smears of numerous fly strains have been screened for the presence of lamellocytes. I included strains that I used in this work and additional randomly selected lines that were available in our laboratory.

| Strain | Presence of lamellocytes |
|---|---|
| *l(2)gl* (VDRC 51247/GD) | 0 |
| *l(2)gl* (VDRC 51249/GD) | 0 |
| *warts* (VDRC 9928/GD) | 0 |
| *discs large 1* (VDRC 41134/GD) | 0 |
| *fat* (VDRC 9396/GD) | + |
| *scribbled* (VDRC 105412/KK) | 0 |
| *PTEN* (VDRC 35731/GD) | + |
| *Csk* (VDRC 32877/GD) | 0 |
| *GP 150* (VDRC 899/GD) | 0 |
| *UAS-p35* | + |
| *UAS-GFP* (2nd chromosome) | + |
| *UAS-GFP* (3rd chromosome) | 0 |
| *UAS-grim* | + |
| *UAS-Ras$^{V12}$* (Bl 4847) | + |
| *Bx$^{ms1096}$-GAL4* (Bl 8860) | - |

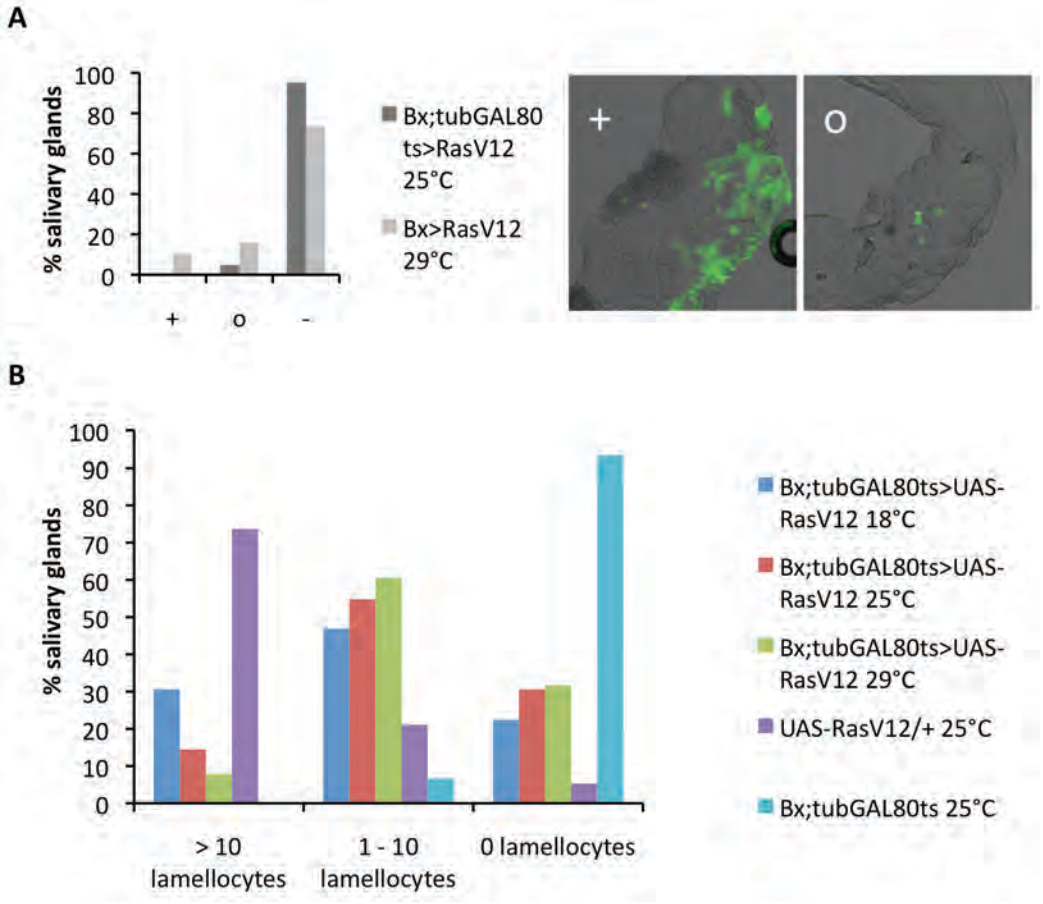


**Figure 23. Lamellocytes adhere to Ras$^{V12}$ salivary glands.** (A) Image-analysis based quantification of lamellocyte adherance to salivary glands: three phenotypes could be distinguished on Ras$^{V12}$ salivary glands (- = no lamellocytes detected; o = individual lamellocytes or small clusters of this cell type; + = patch of lamellocytes, individual cells cannot be distinguished). Salivary glands from larvae raised at 25°C ($n$=42) and 29°C ($n$=76) were compared (*BxGAL4*; *tubGAL80$^{ts}$* > Ras$^{V12}$). At 29°C, 10.5 % of salivary glands were of the (+) phenotype while none of the glands at 25°C showed a (+) phenotype. (o): 15.8 % (29˚C) versus 4.8% (25˚C) and (-): 73.7 (29˚C) versus 95.2 % (25˚C). (B) quantification of lamellocytes in circulation. 93.3 % of *Bx$^{ms1096}$ GAL4*; *tubGAL80$^{ts}$* larvae ($n$= 15) did not contain lamellocytes while 73.7 % of heterozygous *UAS-Ras$^{V12}$*/+ larvae ($n$= 19) contained ≥ 10 lamellocytes in circulation. Larvae with Ras$^{V12}$ aberrant tissue at 29°C ($n$= 103) had 3.9 times lower lamellocyte counts (≥ 10) than genetically equal larvae at 18°C ($n$= 49) and 1.8 times lower counts than respective larvae at 25 ˚C ($n$= 62).

### 4.2.5 Plasmatocyte attachment correlates with cell death in Ras$^{V12}$ salivary glands

Having established that Ras$^{V12}$ salivary glands are encapsulated, I assessed physiology of the aberrant tissue. An indicator that viability of Ras$^{V12}$ expressing glands is altered was the finding that GFP was unevenly distributed compared to wt glands (Figure 17, B and C). An interesting finding was that Ras$^{V12}$ glands stain in part propidium iodide (proI) positive, indicating necrotic or late-apoptotic cell death (see paragraph 3.12). The staining pattern roughly negatively correlated with GFP labeling. This seemed contradictory to previous findings reporting that Ras$^{V12}$

expression in salivary glands prevents degradation of the tissue (Berry and Baehrecke 2007). The propidium iodide staining pattern positively correlated with hemocyte adherence (Figure 25; A). Hemocytes might hence either be the inducers of cell death or alternatively are attracted by dying cells.

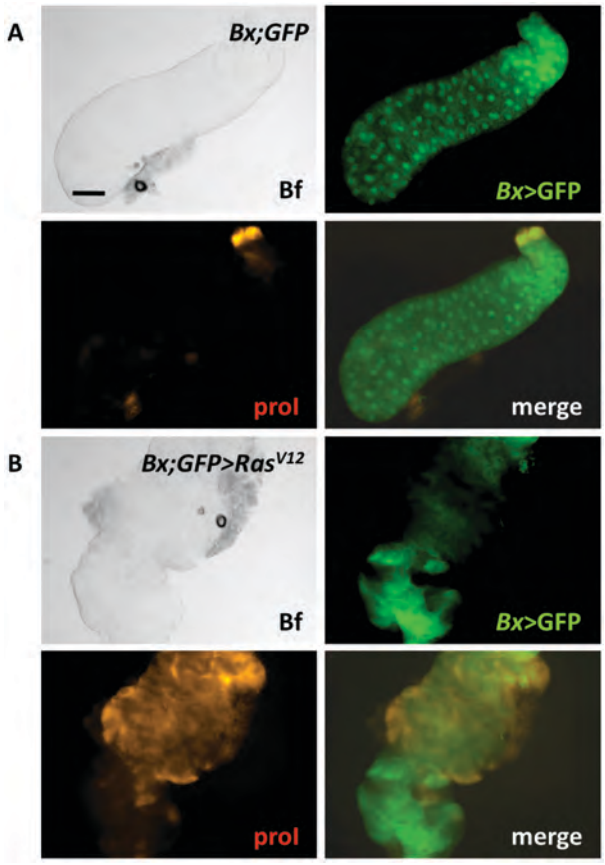


**Figure 24. Ras$^{V12}$ expression is salivary glands induces necrosis.** Salivary glands have been labeled by GFP coexpression (*Bx GAL4* > *UAS-GFP*, short: *Bx>GFP*). Propidium iodide (proI) was used to stain necrotic cells. Bright field projections (Bf) of the glands are shown for comparison. (A) In GFP + salivary glands (*Bx;GFP*), proI staining was observed at the tip of the gland and in the surrounding fat body. GFP was evenly distributed. (B) In contrast proI stained a large part of a GFP Ras$^{V12}$ salivary gland (*Bx GAL4*; *UAS-GFP* > *UAS-Ras$^{V12}$* - short: *Bx;GFP*>Ras$^{V12}$). Most GFP signal was lost in this part indicating that GFP can be used as a viability marker (merge). Scale bar: 100 µm.

Interestingly, *scrib*$^{-/-}$ clones in imaginal tissue undergo Eiger-JNK mediated apoptosis via auto- or paracrine signaling (Igaki, Pastor-Pareja et al. 2009). Furthermore Ras$^{V12}$ expressing cells were demonstrated to induce apoptosis in imaginal tissues (Karim and Rubin 1998), while cell-intrinsic Ras expression promotes cell survival and growth (Kurada and White 1998; Prober and Edgar 2000). Furthermore, the concept that apoptosis represents a barrier to tumor development has been firmly established (Evan and Littlewood 1998; Adams and Cory 2007).

I therefore tested if apoptosis, measured as caspase activity detected by FLICA (fluorophor-conjugated inhibitor of active caspases) staining, occured in hemocyte-infiltrated tumor-like Ras$^{V12}$ salivary glands. I could not detect caspase activity in hemocytes but salivary gland cells stained in part FLICA positive and nuclei of the respective cells showed clear signs of DNA fragmentation (Figure 25; B).

Salivary glands that expressed the apoptosis-inducer grim at low levels (*Bx-GAL4*; *tub-GAL80*$^{ts}$ > *UAS-grim*) were smaller than their wt and Ras$^{V12}$ expressing counterparts. Crucially though, grim expressing salivary glands did not attract hemocytes, suggesting that apoptosis-associated signals do not attract hemocytes to aberrant tissues (Figure 25, E).

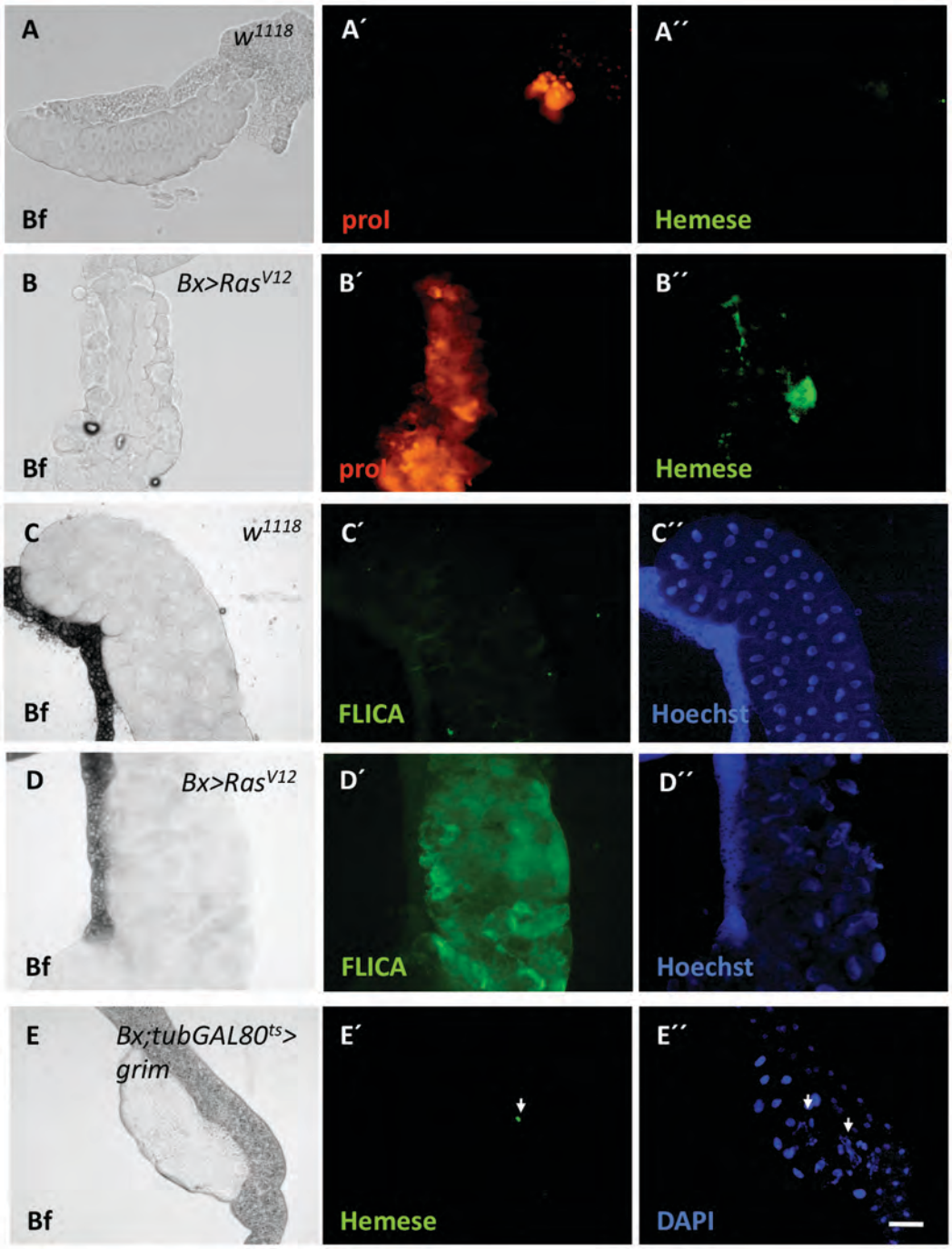


**Figure 25. Adhering hemocytes and cell death of aberrant salivary gland cells correlate.** (B-B´´) Ras$^{V12}$ glands (*Bx GAL4>UAS-Ras$^{V12}$*) stained propidium iodide positive (prol) indicating a necrotic or late apoptotic cell death. This correlated with hemocyte adherance (Hemese). (A-A´´) Wt glands (*w$^{1118}$*) showed little proI staining at the base of the gland, putatively due to mechanical injury during preparation since hemocytes could not be detected (B´´). (C – D´´) Detection of active caspases (FLICA) as a read-out for apoptosis: Ras$^{V12}$ salivary glands (D) contained active caspases (D´), which were absent in salivary glands from naive wt larvae (C´). Nuclear staining (Hoechst in D´´) revealed DNA fragmentation in caspase-positive tissue sections while nuclei appeared intact in the wt (C´´). (E – E´´) Inducing apoptosis in salivary glands *in vivo* (*Bx GAL4>tub GAL80$^{ts}$ > UAS-grim*) rarely resulted in hemocyte attachment (arrow in E´). DNA fragmentation (DNA stained with DAPI) is observed (white arrows in E´´). Scale bar: 100 µm.

### 4.2.6 Partial tumor necrosis factor (Eiger) deficiency results in larger $Ras^{V12}$ salivary glands and enhanced encapsulation

Having established that $Ras^{V12}$ salivary glands stain positive for apoptotic markers I investigated whether impairing apoptosis had an impact on the growth of aberrant salivary glands.

TNF-alpha has been discussed as a cytokine secreted by vertebrate tumors that provokes an inflammatory response and recent studies in *Drosophila* suggest that apoptosis is induced via auto- and paracrine Eiger-JNK signaling (Igaki, Kanda et al. 2002; Moreno, Yan et al. 2002). For instance, Eiger has been shown to be required for JNK-mediated induction of apoptosis in $scrib^{-/-}$ tumor cells in imaginal discs (Igaki, Pastor-Pareja et al. 2009).

I investigated whether Eiger has an impact on the growth of aberrant salivary glands by inducing $Ras^{V12}$ in salivary glands in an *eiger* hypomorph mutant background.

$Ras^{V12}$ salivary glands grew 45.7% larger in *eiger*-mutant larvae compared to salivary glands in a wt background supporting the idea that Eiger signaling also limits growth of aberrant salivary glands. Impairing Eiger function did not have an impact on the growth of wt glands and hence did not seem to be required for normal development. In addition, hemocyte attachment increased more than twofold, arguing that Eiger indeed had an impact on $Ras^{V12}$ aberrant cell survival leading to larger glands that continue to secrete cytokines, which in turn attract more hemocytes (Figure 28; A and B). Supporting this idea, encapsulation (i.e. lamellocyte adherance) was enhanced by 20% compared to $Ras^{V12}$ salivary glands in a wt background when Eiger receptor *Wengen* was co-knocked down in $Ras^{V12}$ salivary glands (*Bx-GAL4*; *UAS-wgn*$^{RNAi}$ > *UAS-Ras*$^{V12}$: $n$= 34 versus *Bx-GAL4* > *UAS-Ras*$^{V12}$: $n$= 35). It has further been demonstrated that Eiger-Wengen mediated apoptosis of $scrib^{-/-}$ and $l(2)gl^{-/-}$ cells is endocytosis dependant and can be impaired by expressing dominant-negative Rab5 ($Rab5^{DN}$) in these cells. In line with my hypothesis that impairing apoptosis in salivary glands results in increased encapsulation I observed melanisation on salivary glands of larvae in which *l(2)gl* was knocked down and $Rab5^{DN}$ coexpressed under the control of *Bx GAL4*. I never detected melanisation when *l(2)gl* alone was knocked down.

### 4.2.7 An immune mutant, which lacks functional crystal cells enhances non-melanotic encapsulation and growth of $Ras^{V12}$ salivary glands

Having observed encapsulation-associated melanization as a response against *Drosophila* tumors and wing melanization upon *l(2)gl* knockdown and $Ras^{V12}$ expression in wing discs, I investigated if compromising the PPO activating system had an effect on aberrant growth. Wandering stage larvae with the *Black cells*$^{1}$ (*Bc*$^{1}$) mutation lack functional crystal cells and are therefore completely deprived of PO activity in the hemolymph (Rizki, Rizki et al. 1980). Furthermore, hemolytic capsules around parasitoid eggs do not melanize and harden in *Bc*$^{1}$ larvae (Rizki and Rizki 1990). *Bc*$^{1}$ mutants that are homozygous or heterozygous for the mutation have preactivated and hence non-functional crystal cells, which in turn are phagocytosed by plasmatocytes. Furthermore, lamellocyte differentiation is observed in *Bc*$^{1}$ mutants (Lanot et al., 2001). When comparing blood smears I found that hemocyte numbers appeared to be higher in *Bc*$^{1}$ larvae compared to wt (Figure 27, B). Strikingly, $Ras^{V12}$ salivary glands in a *Bc*$^{1}$ mutant background grew larger than in wt larvae with an uncompromised immune system (Figure 28, A) despite increased plasmatocyte numbers and lamellocyte proliferation in circulation and although encapsulation was more pronounced in the mutant (Figure 28, B). This observation indicated that cytotoxic molecules and/ or capsule hardening as the consequences of PO activity normally restrict overgrowth of aberrant salivary glands. Supporting this finding, RNAi knock down of *l(2)gl* in *Bc*$^{1}$ mutant larvae (*Bx*$^{ms1096}$;*Bc*$^{1}$ > *l(2)gl*$^{RNAi}$) led to a complete loss of wing melanization in 55% of the flies compared to 11% in a wt background (*Bx*$^{ms1096}$ > *l(2)gl*$^{RNAi}$). Non-melanized wings were larger than wt and melanized wings, similar to the phenotype observed when *fat* and *warts* were knocked down under *Bx-GAL4* in a wt background (Figure 16, A: *fat* and *warts*). PO hence also appeared to restrict the development of aberrant wings (Figure 26, B).

It has been proposed that PPO expressed by lamellocytes contributes to melanization during encapsulation (Irving, Ubeda et al. 2005). At least in the case for encapsulation of $Ras^{V12}$ salivary glands this did not seem to be the case since salivary glands never melanized in the *Bc*$^{1}$ background despite enhanced encapsulation (Figure 26, A and Figure 28, B).

Interestingly, plasmatocytes that apparently had engulfed aberrantly-activated crystal cells still infiltrated $Ras^{V12}$ salivary glands (Figure 26, A) It has been suggested

that plasmatocytes in *Bc*$^{1}$ larvae are impaired in their phagocytic function (Ayres and Schneider 2008) since melanized bodies, which are engulfed by plasmatocytes, cannot be degraded in the phagosome and hence would prevent or impair the uptake of apoptotic aberrant cells. Whether phagocytosis has a role in clearance of aberrant salivary gland cells remains to be investigated.

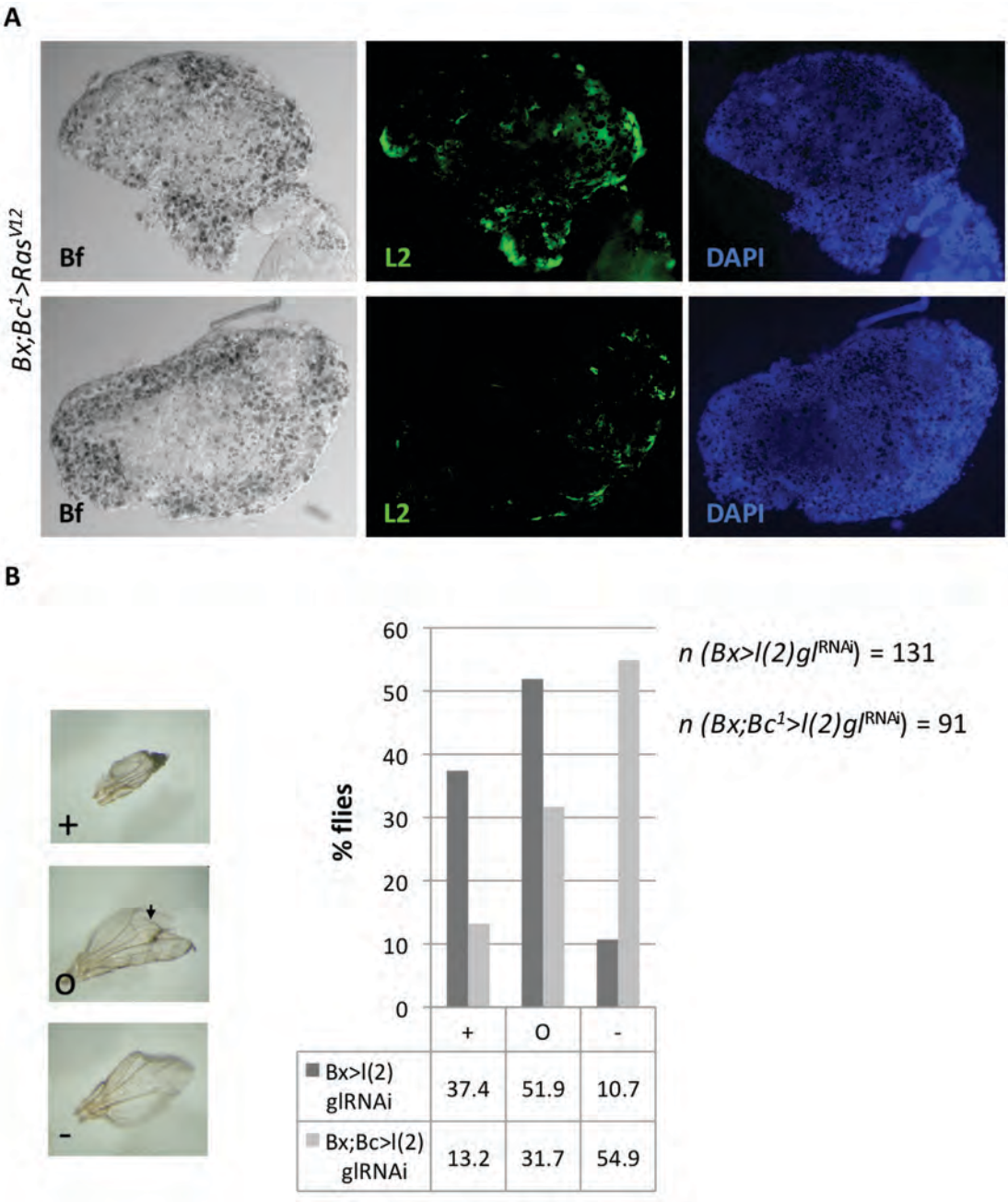


**Figure 26. The melanization of encapsulated Ras$^{V12}$ salivary glands and *l(2)gl$^{RNAi}$* wings requires functional crystal cells.** (A) Ras$^{V12}$ salivary glands in heterozygous *Bc$^{1}$* larvae (*Bx GAL4;Bc$^{1}$>UAS-Ras$^{V12}$*, short: *Bx;Bc$^{1}$*>Ras$^{V12}$) exhibited pronounced encapsulation involving mature L2-positive lamellocytes (L2). DNA was stained with DAPI. Plasmatocytes that have engulfed crystal cells (black spots) also adhered to the gland. The upper row showed a capsule, which formed on an aberrant salivary gland. Depicted in the lower row is a completely encapsulated tissue fragment isolated from the hemolymph. (B) Wing melanization as a result of *l(2)gl* RNAi knockdown was reduced in *Bc$^{1}$* heterozygous mutant flies (*Bx GAL4;Bc$^{1}$ > UAS-l(2)gl$^{RNAi}$*, short: *Bx;Bc$^{1}$>l(2)gl$^{RNAi}$*; n= 91) compared to the wt background (*Bx>l(2)gl$^{RNAi}$*; n= 131). Wings with pronounced melanization and reduced wing size are referred to as +; melanotic spots in the central wing area (arrow) are defined as O; no melanisation: –. The values in the table represent percentages.

The effect of the immune system on tumor growth is controversial. Numerous studies suggest that immune responses can be both tumor promoting and tumor growth antagonizing. In mammals, tumor-associated macrophages (TAMs) are discussed to foster tumorigenesis by providing MMP to breakdown the ECM that would otherwise restrict tumor growth (Kessenbrock, Plaks et al. 2010).

In *Drosophila*, larvae with constitutively activated Toll signaling express MMP in hemocytes (Irving, Ubeda et al. 2005). Moreover, melanization can activate Toll signaling (Tang, Kambris et al. 2008). Having observed that salivary glands grew larger in $Bc^{1}$ larvae, which contain melanotic bodies in their hemolymph I reasoned that MMP might be expressed in hemocytes, thereby potentially contributing to the observed overgrowth of salivary glands.

$Ras^{V12}$ was expressed in salivary glands in larvae with GFP-labeled hemocytes (*Bx>tep1-GFP*; $Ras^{V12}$). Dissected glands were detected with MMP1-antibody, which in turn was detected with Cy3-conjugated secondary antibody. Most MMP appeared to originate from the glands and not from adhering hemocytes and MMP was not detected in circulating hemocytes. In contrast, blood smears of *Bx*; $Bc^{1}$ and *Bx;* $Bc^{1}$*>* $Ras^{V12}$ larvae revealed that virtually all hemocytes expressed the ECM degrading enzyme, thereby putatively contributing to overgrowth of the glands (Figure 27).

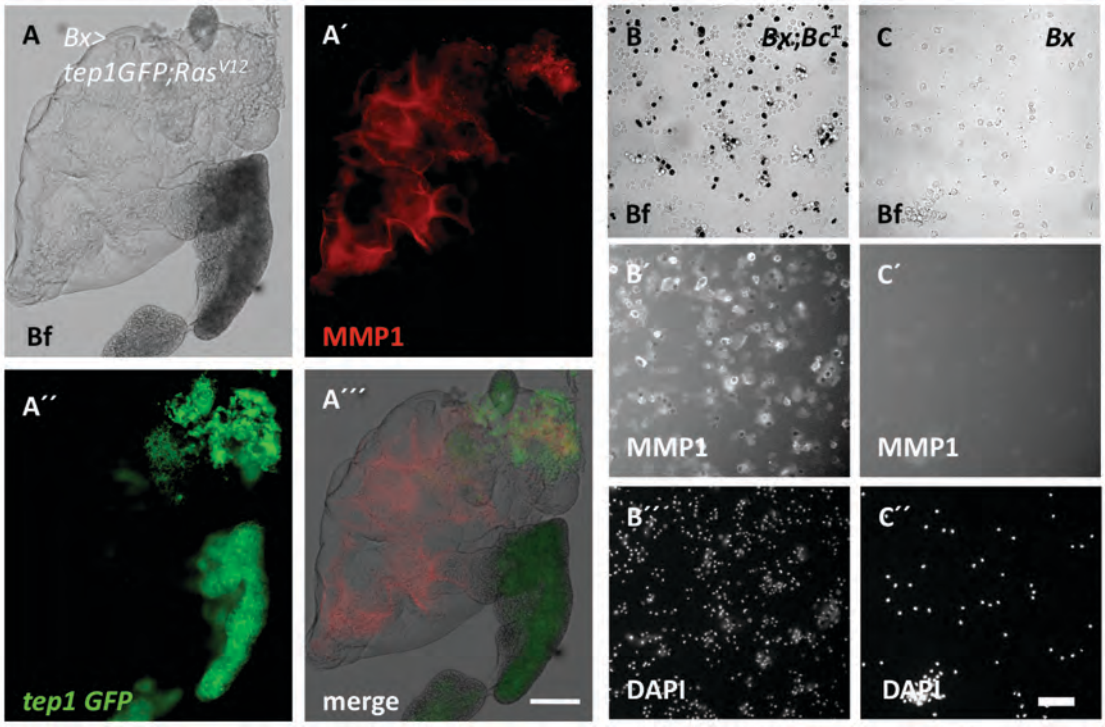


**Figure 27. MMP1 expression can be induced in hemocytes.** (A) MMP was mostly expressed in aberrant salivary glands in *Bx GAL4>tep1GFP*; *UAS-Ras$^{V12}$* larvae. Shown are bright field (Bf), epifluorescence images (MMP1 and *tep1 GFP*) and the corresponding merged view of the three channels (merge). (B) Blood smears of *Bx GAL4;Bc$^{1}$* (short *Bx;Bc$^{1}$*) and *Bx GAL4*;+ (*Bx*) larvae. MMP1 was detected in hemocytes in larvae carrying the *Bc$^{1}$* mutation (B´) but not in hemocytes of the control *Bx GAL4* driver larvae (C´). Note that hemocyte numbers in *Bx GAL4;Bc$^{1}$* larvae (B´´; nuclear DNA stained with DAPI) were higher than in the control (C´´).

A method to quantify overgrowth and hemocyte attachment

The size of salivary glands was measured as described in paragraph 3.14.1. In order to precisely quantify the hemocyte attachment to salivary glands, I developed a method to screen and measure the total area covered by Hemese-positive hemocytes, which in turn were detected by FITC-conjugated anti-Hemese antibody (see paragraph 3.14.2). Clearly, the level of hemocyte attachment was elevated when Ras$^{V12}$ expression was maximum induced in salivary glands at 29 ˚C compared to weaker induction at 23˚C. Enhanced infiltration also coincided with amplified outgrowth of salivary glands (Figure 28; A and B). Comprising Eiger signaling resulted in a significant increase of both organ size and hemocyte attachment compared to parental controls and Ras$^{V12}$ salivary glands in a wt background. This trend, albeit more pronounced, was also observed when RasV$^{12}$ was induced in the immunodeficiency mutant *Bc$^{1}$*. Different from Ras$^{V12}$ glands, knockdown of *l(2)gl* in the *Bc$^{1}$* background did not have a significant effect on gland size and hemocyte attachment compared to *l(2)gl* knockdown in a wt background.

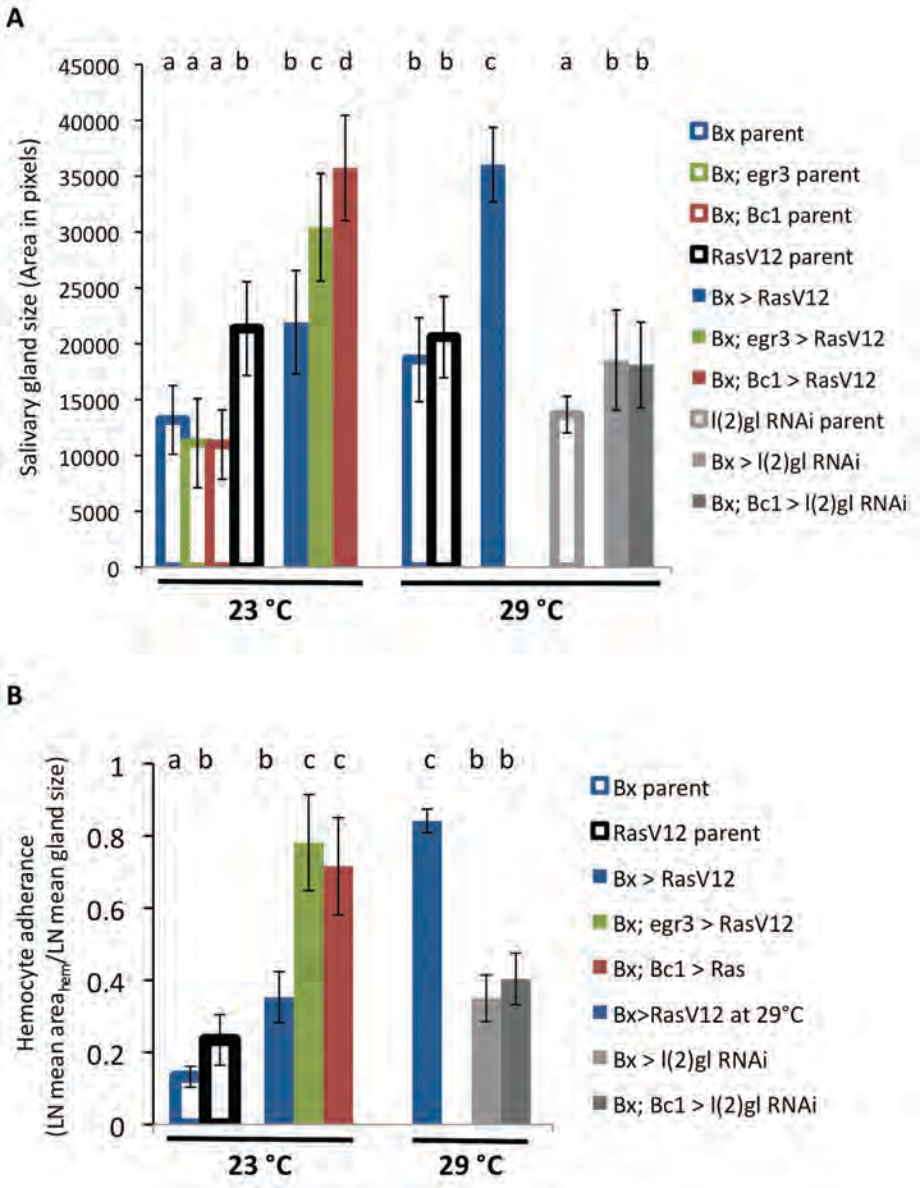


**Figure 28. Aberrant tissue growth and encapsulation are enhanced in an Eiger mutant background and in larvae lacking functional crystal cells.** (A) Average salivary gland size has been quantified (in pixels) in imageJ as described in paragraph 3.14.1. Salivary gland size was modified by temperature-dependent induction of $Ras^{V12}$: the organ size increased with elevated $Ras^{V12}$ expression at 29°C (*n*= 25) compared to 23°C (*n*= 59; p = 5E-9). An increase in gland size was also observed when $Ras^{V12}$ was induced in a $Bc^{1}$ (*n*= 20) and an $egr^{3}$ hypomorph background (*n*= 20) compared to induction in the wt background ($Bc^{1}$: p = 4E-8, $egr^{3}$: p = 9E-7) and compared to the parental lines (*UAS-*$Ras^{V12}$ parent, *n*= 18; *Bx* parent, *n*= 11; *Bx*;$Bc^{1}$, *n*= 20; *Bx*;$egr^{3}$, *n*= 13). Supporting the result for $Ras^{V12}$, *l(2)gl*$^{RNAi}$ salivary glands (*n*= 28) were significantly larger than the parental controls (*n*= 13). Contrary to $Ras^{V12}$ glands, $Bc^{1}$ (*n*= 11) did not affect the size of *l(2)gl*$^{RNAi}$ glands in comparison to the wt background (*p* = 0.5). Samples that differed insignificantly are labeled with the same letter; they differed from other groups significantly ($p \leq 0.01$). (B) Hemocyte-adherance to aberrant salivary glands was calculated as described in paragraph 3.14.2 ($area_{hem}$: gland area occupied by hemocytes). Relative hemocyte-adherance to $Ras^{V12}$ glands was enhanced in $Bc^{1}$ (*n*= 23) and $egr^{3}$ (*n*= 12) mutant backgrounds compared to the wt (*n*= 14) genetic background ($Bc^{1}$ *p* = 4E-08 ; $egr^{3}$ *p* = 9E-07). Likewise, hemocyte-adherance increased with amplified $Ras^{V12}$ induction (*Bx*>$Ras^{V12}$ at 29°C [*n*= 12] versus 25°C [*n*= 14]: *p* = 5E-09). Hemocyte infiltration was not significantly enhanced when $Ras^{V12}$ was induced at 25 °C compared to the parental $Ras^{V12}$ line (*n*= 23; p= 0.19). Compared to *Bx GAL4* (n= 11), *l(2)gl* RNAi knockdown (*n*= 15) attracted hemocytes. The $Bc^{1}$ mutation (*n*= 13) did not affect hemocyte-infiltration of *l(2)gl*$^{RNAi}$ glands.

### 4.2.8 Aberrant salivary glands induce expression of humoral Tep1 in the fat body

A first indication that altered self is also sensed by the humoral branch of the immune system originated from the observation that Tep1, which is constitutively expressed in larval hemocytes was induced in the fat body when expression of $Ras^{V12}$ was induced in wing imaginal discs and salivary glands or in salivary glands alone. The data was obtained using the *tep1-GFP* reporter strain (described in Figure 8, E). Likewise, *tep1*-driven GFP production was also observed when $Ras^{V12}$ was expressed in the fat body (*5253-GAL4>tep1-GFP*; $Ras^{V12}$; not shown). Tep1 has previously been reported to be fat body induced in septically wounded larvae, i.e. larvae that were pricked with a needle that had been dipped into a pellet containing Gram-negative and Gram-positive bacteria (Lagueux, Perrodou et al. 2000; Irving, Troxler et al. 2001). Moreover, Tep1 was reported to be involved in phagocytosis of fungi by *Drosophila* S2 cells and strikingly *tep1* appears also to be upregulated in larvae that mount encapsulation in response to wasp infection (Wertheim, Kraaijeveld et al. 2005).

Tep1 was also fat body induced in larvae when *l(2)gl* was knocked down in salivary glands and to a lesser extent in $spag^{k12101}$ larvae that encapsulate self tissue but not in larvae that express the apoptosis-inducer grim in salivary glands and wing discs (Figure 29; B). In conclusion, endogenous stimuli that are not apoptosis-related can induce Tep1 expression in the fat body in the absence of microbial, foreign elicitors. It has been suggested that the putative opsonin is regulated by JAK/STAT signaling (Lagueux, Perrodou et al. 2000). Supporting this hypothesis, it has been shown previously that the JAK/STAT pathway is upregulated in the fat body of larvae with metastatic tumors in imaginal tissue (Pastor-Pareja, Wu et al. 2008).

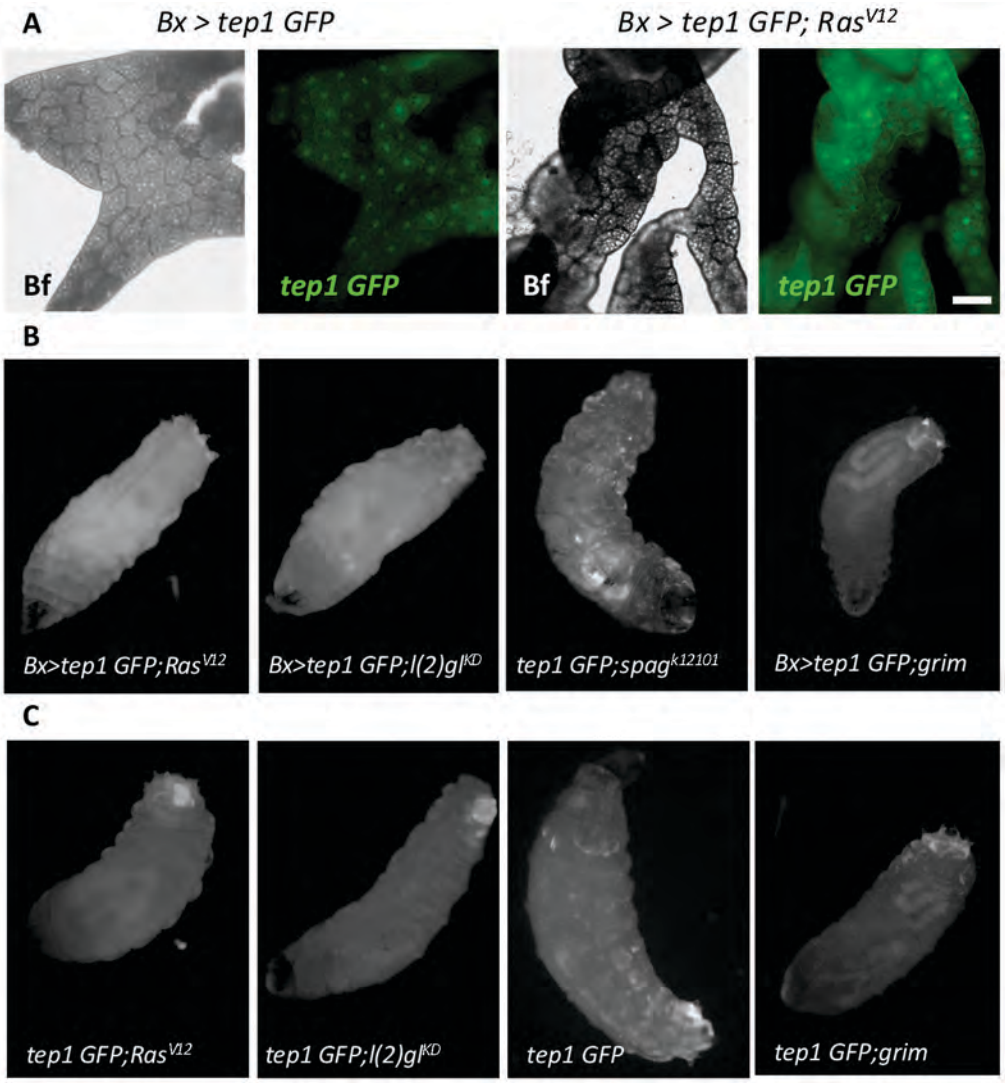


**Figure 29. The humoral effector Tep1 is upregulated in the fat body of larvae that have been immune-challenged by aberrant tissue**. (A) Fat body preparations from tep1-GFP parent and *Bx GAL4 > tep1 GFP;UAS-Ras$^{V12}$* (short: *Bx>tep1 GFP*;Ras$^{V12}$) expressing larvae. Tep1 was induced in the fat body of the Ras$^{V12}$ expressing larva but not in the parental control. (B) Expression of Ras$^{V12}$ and of *l(2)gl* RNAi knockdown in glands alone was sufficient to trigger Tep1 production in the fat body (depicted are grayscale images; light gray tones reflect *tep1*-GFP induction). Some induction was also observed in *spag$^{k12101}$* larvae but not in larvae with stimulated apoptosis in wing imaginal discs and salivary glands *Bx GAL4 > tep1 GFP*; *UAS-grim* (short: *Bx>tep1GFP;grim*) indicating that apoptosis, which occurred in the aberrant tissues was not the trigger for Tep1 expression. (C) Fat body induction was not observed in the parental controls.

# 5 Discussion

## 5.1 Endogenous and microbial factors activate insect PPO

PO-mediated melanization is a key mechanism of immunity in many invertebrates (Cerenius, Lee et al. 2008).

In this work, I have shown that the PO zymogen PPO can be activated by endogenous danger signals as well as foreign, microbial elicitors. Two prominent, bacteria-specific molecules, PGN and LPS are PPO-activating in *Galleria* but not in *Drosophila*. The patterns of PPO activation appear to differ between species or orders (*Lepidoptera* versus *Diptera*). Melanization as the ultimate consequence of PO activity was restricted to clot folds and fibers in two Dipterans while being systemic in the hemolymph of two Lepidopterans. Systemic PPO activation instead of localized melanization could also be triggered in *Drosophila* by depleting a regulatory serpin Spn27A. Possibly, similar inhibiting serpins are present in Lepidopterans at lower concentrations in naïve larvae.

### 5.1.1 Activation of PPO: Dipterans versus Lepidopterans

The activation of PPO apparently differs between insect species however melanization patterns appear to be similar within the same order: both Lepidopterans melanized systemically while melanization remained local in the clot of the two Dipterans. PPO is among the fastest evolving immune genes in *Drosophila* and mosquitoes (Clark, Eisen et al. 2007; Sackton, Lazzaro et al. 2007; Waterhouse, Kriventseva et al. 2007) and its evolution is likely triggered by pathogens that the respective species encounters. Hence, if two species share the same pathogens they might have evolved similar modes of PPO activation. Whether this is true for *Pararge* and *Galleria* and for *Drosophila* and *Anopheles*, respectively remains to be shown. At least for *Drosophila* and *Anopheles* this seems unlikely as they have significantly different feeding habits and the developments larvae develop in are different: *Anopheles* larvae require an aquatic milieu while *Drosophila* larvae naturally feed on decaying fruit.

Unmistakably, this study demonstrates that PPO activation differs between insect species and care must thus be taken when deducing general conclusions about PPO activation from studies based on one species only. This work however supports the idea that PO in *Drosophila* does not specifically target pathogens since the enzyme was mostly activated by endogenous signals as opposed to microbial patterns in the moth *Galleria*. However, PPO in *Drosophila* flies and larvae *in vivo* might not be activated by endogenous elicitors exclusively as bacterial sepsis has been shown to indirectly contribute to PPO activation in a transcription dependent manner (Ligoxygakis, Pelte et al. 2002). Moreover, certain bacteria and wasp species actively inhibit melanization in *Drosophila* larvae by secreting inhibitors that target the PPO cascade and MP2 mutant larvae that fail to activate PPO were more susceptible to septical and natural infections indicating that melanization is harmful for invading pathogens (Tang 2009).

Melanization can have fatal consequences for larvae because of generally toxic byproducts and because melanotic bodies cannot be broken down by the insect thus potentially presenting a burden. The activation of PPO must therefore be tightly controlled. Due to a larger body volume *Galleria* and *Pararge* larvae might posses a higher capacity/tolerance for melanization than *Drosophila* does.

### 5.1.2 Model of PPO activation in the clot of *Drosophila* larvae

Based on the finding that melanization can be inhibited in the clot by Spn27A I propose that in the clot the PO zymogen PPO is proteolytically activated. Following small wounds, crystal cells are activated and rupture, PPO is released, solubilized and forms complexes with activating proteases and aminophospholipids such as PS and PE present on dying cells. These complexes form on the clot, become separated from the hemolymph inside clot folds where PO activity ultimately leads to melanization. The presence of serine protease inhibitors including Spn27A in the hemolymph prevents systemic melanization. The hydrophobic clot presents a diffusion barrier for Spn27A thus allowing for PPO activation.

In case bacterial entrapment by the clot is insufficient and the hemolymph becomes infected, microbial products trigger the activation of immune genes and activate a humoral response, including breakdown of Spn27A and systemic activation of PPO.

The situation appears different in *Galleria*, where PPO activation is more systemic and sensitive to microbial elicitors before the onset of any transcriptional activation.

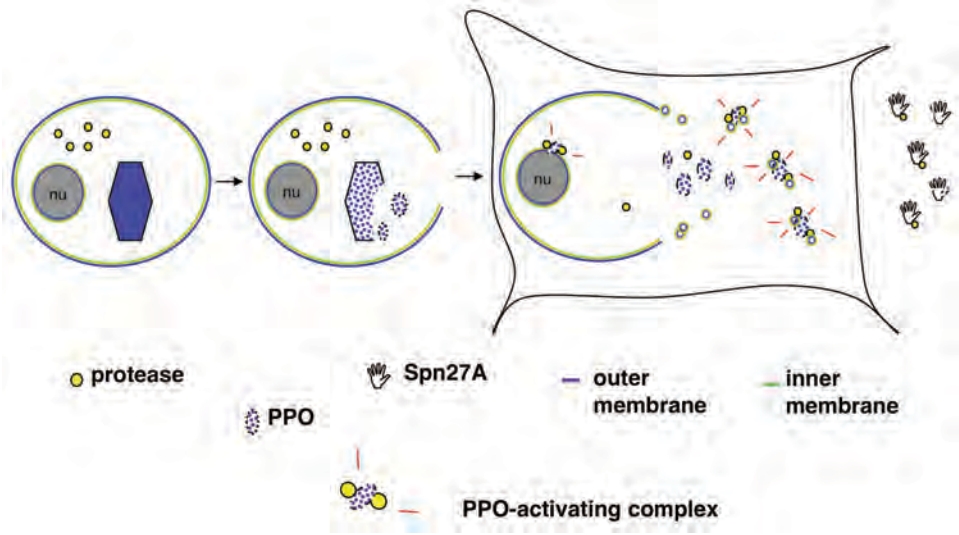


**Figure 30. Model of prophenoloxidase (PPO) activation in the *Drosophila* clot.** After release from crystal cell, PPO is activated in a complex containing activating proteases and inner-membrane lipids released during crystal cell rupture. Inside the clot, PPO complexes have limited access to Serpin 27A (Spn27A) due to limited diffusion of the serpin into the clot matrix. Outside of the clot the PPO-activating proteases are inhibited by Spn27A. nu: nucleus. Black line: clot border. Figure adapted from (Bidla, Hauling et al. 2009).

### 5.1.3 Aberrant apoptosis can activate the PPO cascade

PPO and as a consequence melanization could be activated by inducing apoptosis in a subpopulation of hemocytes. However, no melanization was observed when apoptosis was induced in other tissues, including imaginal discs, the larval fat body and the salivary glands. In vertebrates, inner membrane lipids that are exposed during apoptosis provide an assembly platform for the enzymatic clotting cascade (Morrissey, Pureza et al. 2008). Cells that undergo apoptosis in the hemolymph would be readily "visible" for clotting enzymes. The situation seems to be different for apoptosis in other tissues. Organs in multicellular organisms are lined and thereby separated from the extracellular space by a layer consisting of structural proteins and signaling molecules termed extracellular matrix (ECM). Possibly, the ECM interferes with immune-recognition of apoptotic signals present on the surface or secreted by death-induced cells.

It is also conceivable that melanization observed in larvae with apoptosis-induced hemocytes is actually the result of an auto-encapsulation process. In such larvae

hemocytes form large melanotic aggregates that likely consist of multiple hemocytes and possible different hemocyte subtypes since lamellocytes are also observed in bleeds of UAS-grim larvae. The absence of melanization might be explained by the fact that encapsulation is a process that can require days to complete (Williams 2007) thus possibly exceeding the larval stage.

## *5.2* An innate immune response to tumor-like tissue in *Drosophila melanogaster*

In this work, I have shown that *Drosophila* larvae elicit a complex defense reaction comprising both the humoral arm and the cellular arm of the immune system against aberrant tissue in the form of tumor-like salivary glands.

In *Drosophila* aberrant tissue has been described as tumorous when this tissue a) grows significantly larger than it unaltered counterpart and b) the tissue lost its structure/organization. Malignancy and invasive growth has been ascribed to tumors that formed metastases at distal sites from the original tumor site. These metastases could be detected because induced tumors where simultaneously GFP-labeled. I have shown that expression of a single oncogene, constitutively activated $Ras^{V12}$ is sufficient to induce tumor-like salivary glands that invade the surrounding hemolymph putatively by proteolytically breaking down the ECM. This is in contrast to tumors of the eye imaginal disc and glial cells that require two alterations (Ras activation and depletion of a tumor suppressor) to become invasive (Brumby and Richardson 2003; Pagliarini and Xu 2003; Read, Cavenee et al. 2009).

My results suggest that encapsulation is the general defense mechanism against aberrant tissue consisting of larger polytene cells such as salivary glands and fat body. Furthermore, my data indicate that the immune response can be both inhibiting and supporting aberrant tissue growth. Third, my data suggest that as in vertebrate tumors, dTNF/ Eiger signaling regulates growth of tumor-like $Ras^{V12}$ salivary glands.

### 5.2.1 Inducing tumorous overgrowth with the GAL 4 system – Expression of $Ras^{V12}$ in larval salivary glands is sufficient to induce invasive overgrowth

In vertebrates, tumor development requires multiple mutations before metastasis can occur. Also in *Drosophila*, current models of tumor formation assume that at least two mutations are necessary to transform larval tissue into invasive tumors as exemplified by tumors originating from imaginal discs that require Ras activation and depletion of a tumor suppressor of the Scribbled polarity module (Brumby and Richardson 2003) and glial cells that in addition to activated Ras require PTEN depletion to become invasive (Read, Cavenee et al. 2009).

In contrast to a previous study on metastatic behaviour of tumors in the eye imaginal disc in *Drosophila* larvae (Pagliarini and Xu 2003) I have shown that at least in larval salivary glands over-expression of a single oncogene, namely $Ras^{V12}$ results in overgrowth of the gland and MMP expression, putatively facilitating the escape of gland tissue into the surrounding hemolymph. *Drosophila* salivary gland tumors hence represent a genetically very simple and therefore highly reproducible system to study the impact of exogenous factors such as the immune system on tumor growth.

Virtually all vertebrate malignancies are associated with tumor-borne MMP expression. MMPs are secreted into the surrounding environment where they catalyze the breakdown of ECM components such as collagen IV/ viking. Previously, it has been shown in *Drosophila* that MMP dependant remodeling of the ECM is required for tumor invasion of imaginal discs (Pagliarini and Xu 2003; Uhlirova and Bohmann 2006; Srivastava, Pastor-Pareja et al. 2007).

MMP expression itself might explain in parts the immune response to the tumor: Breakdown products of the ECM due to MMP activity have also been shown to be inducers of humoral immunity, i.e. AMP expression in *Galleria* larvae (Altincicek and Vilcinskas 2006). I have shown that MMP expression in salivary glands also induces a cellular response as hemocytes adhere. However, MMP expression alone is unlikely to cause the infiltration observed in $Ras^{V12}$ salivary gland tumors as only a few hemocytes adhered to salivary glands expressing an MMP2 transgene. This confirms a previously published result (Pastor-Pareja, Wu et al. 2008) and is in line with data from vertebrate studies showing that ECM fragments attract leukocytes to wounds (Korpos, Wu et al. 2010).

### 5.2.2 Aberrant Salivary glands induce TEP1, opening a new path towards functional understanding of the TEP family

Until today, no immunological function of TEPs has been demonstrated in the fly *in vivo*. In *Anopheles* TEP1 (aTEP1) directly target *Plasmodium* ookinetes by binding to them, thereby facilitating killing of the parasite (Blandin, Shiao et al. 2004). Furthermore, aTEP1 binds to both Gram positive and Gram negative bacteria in adult mosquitoes and is required for efficient phagocytosis of these bacteria (Blandin and Levashina 2004). However, most *Drosophila* TEPs (dTEPs) seem to share little overall homology with aTEP1 and other mosquito TEPs (except for dTEP3 which is phylogenetically similar to aTEP2 and aTEP15). The three secreted dTEPs (TEP 1,2 and 4) have apparently evolved independently from its counterparts in *Anopheles* (Blandin and Levashina 2004). Thus conclusions about their function drawn from *Anopheles* studies have to be taken with caution. Supporting the opsonin hypothesis, TEPs enhance phagocytic uptake of yeasts and bacteria in *Drosophila* S2 cells *in vitro*. Different TEPs seem to be specific for different microbes: TEP2 was reported to be required for efficient phagocytosis of *Escherichia coli* (but not of two fungi – *Staphylococcus aureus* and *Candida albicans*) while TEP3 exclusively improved phagocytosis of *S. aureus*. TEP6 (Mcr) is required for phagocytosis of *C. albicans* only (Stroschein-Stevenson, Foley et al. 2006).

In vertebrates, deposition of TEP1-like C3 as a consequence of complement activation is a late event during apoptosis and occurs as part of opsonisation, which is required for successful uptake of apoptotic cells by macrophages (Gaipl, Kuenkele et al. 2001; Ciurana, Zwart et al. 2004; Zwart, Ciurana et al. 2004).

C3-like dTEP1 and possibly other dTEPs might similarly function as opsonins, facilitating uptake of aberrant tissue fragments. Interestingly, the induction of dTEP1 along with dTEP2 and dTEP4 has also been shown in larvae that mount encapsulation against eggs of the parasitoid wasp *Asobara tabida* (Wertheim, Kraaijeveld et al. 2005). Yet no function has been assigned to TEPs in parasitoid infections and encapsulation or in infection studies in general so far.

The tumor model that I describe along with the methods to measure tumor size and hemocyte adherence of the same gland might provide a model to investigate TEP function *in vivo* and in a broader sense might also be useful to study the link between complement and cancer in the fly.

In *Anopheles*, it has further been demonstrated that Tep1 requires additional factors, LRIM1 and APL1C to form a complex that mediates ookinete killing (Povelones, Waterhouse et al. 2009). Although there are no clear homologues in *Drosophila*, analogous factors might exist that dTEPs work in concert with.

Tep1 is induced by JAK/STAT signaling and this signaling pathway was also shown to be activated in larvae with tumors in eye imaginal discs putatively via cytokines of the unpaired family. Whether unpaired is expressed and released by salivary gland tumors or whether other cytokine-like molecules are involved in JAK/STAT activation in the fat body remains to be shown.

Vertebrate complement factors C4/C4/C5 are cleaved into two fragments, one large fragment that covalently binds to the target surface and one smaller fragment, which functions as a chemo-attractant to recruit blood cells to the site of complement activation. It is tempting to speculate that C3-like dTEP1, similar to vertebrate C3, is also involved in hemocyte recruitment.

### 5.2.3 Encapsulation - the cellular immune response against $Ras^{V12}$ salivary glands and fat body tumors

Melanotic encapsulation can be induced by injection of oil droplets into larvae of *D. melanogaster* and *D. subobscura* indicating that lipids or charges and object size are sufficient triggers of this type of cellular immunity (Meister 2004; Eslin and Doury 2006).

In this work I have shown that phospholipids can induce melanization in hemolymph preparations and that the expression of $Ras^{V12}$ in salivary glands and fat body results in infiltration with plasmatocytes, lamellocytes and crystal cells, ultimately leading to partial encapsulation of the tissue similar to what is observed in class 1 melanotic mutants *spag*$^{k12101}$ and *kurtz* (Watson, Johnson et al. 1991; Minakhina and Steward 2006).

It has been shown previously that *Drosophila* can encapsulate damaged transplanted tissue and undamaged tissue from distant *Drosophilid* species (Rizki and Rizki 1983). However, these experiments were performed *in tu(1)sz*$^{1}$ larvae, a mutant which exhibit a constitutive auto-encapsulation response. Transplantation experiments in wt larvae could not confirm the results (Rizki and Rizki 1980) and hence more

recently it has been suggested that encapsulation of self was an artifact due to the genetic background as tumors in imaginal discs did not trigger lamellocyte attachment (Pastor-Pareja, Wu et al. 2008) and plasmatocyte infiltration but not encapsulation was reported for *scrib*$^{-/-}$ salivary gland tumors in homozygous *scrib* mutant larvae . One explanation for the absence of lamellocyte on salivary gland tumors in *scrib*$^{-/-}$ larvae might be that lamellocyte adherance requires a primary layer of plasmatocytes, which can only form if plasmatocytes can form septate junctions. The formation of septate junctions however relies on the scribbled complex (Bahri, Wang et al. 2010), and would thus be impaired in *scrib*$^{-/-}$ mutants.
Confirming previous studies, I did not observe encapsulation of Ras$^{V12}$ imaginal discs.

### 5.2.4 Salivary glands versus imaginal discs

Objects that are too large to be phagocytosed by plasmatocytes such as parasitoid eggs or lipid droplets are encapsulated. Secretory cells of larval salivary glands and fat body cells are significantly larger in volume than both imaginal disc cells and hemocytes (SG nuclei are 15x larger than hemocyte nuclei). Thus they are probably too large to be phagocytosed. Moreover, the secretory cells of salivary glands are more metabolically active due to their polytene nuclei than cells with diploid nuclei. They hence might also express and secrete immune-stimulatory signals at a higher rate than tissue consisting of diploid cells such as imaginal discs.
In contrast, cells of imaginal discs are similar in size compared to hemocytes. Imaginal discs are capable of compensatory apoptosis. Clones or patches of cells within wing imaginal discs that express activated oncogenic Myc (Secombe, Pierce et al. 2004) or Ras (Karim and Rubin 1998) induce cell death in surrounding cells thus wing imaginal discs posses a built-in mechanism to antagonize oncogenic overproliferation and conversely wt cells can even induce cell death in clones that are homozygous for tumor suppressors of the apicobasal polarity module (Igaki, Pastor-Pareja et al. 2009; Menendez, Perez-Garijo et al. 2010; Tamori, Bialucha et al. 2010). Compensatory apoptosis of tumor cells might prevent hemocyte infiltration. Perhaps, organs with less plasticity, i.e. organs that are fully differentiated do not posses these compensatory mechanisms and are therefore more prone to enter an aberrant cell fate that can ultimately result in tumorigenesis.

### 5.2.5 Encapsulation and PO

Interestingly, encapsulation of $Ras^{V12}$ salivary glands is shifted more towards encapsulation in larvae that are PO depleted. There is evidence that PO is important in defense against parasitoid eggs: A serpin from *L. boulardi* specifically targets the PPO cascade in *Drosophila* (Colinet, Dubuffet et al. 2009) and components of this enzymatic cascade are also transcriptionally upregulated in larvae upon infection with this wasp species (Schlenke, Morales et al. 2007) resulting in higher PO activity in the hemolymph (Nappi, Carton et al. 1991). POs and serpins were also reported to be induced in larvae infected with the parasitoid wasp *Asobara tabida* (Moreau, Eslin et al. 2003; Wertheim, Kraaijeveld et al. 2005) and moreover, the parasitoid *Campoletis sonorensis* blocks melanization and encapsulation in a Noctuid moth, *Heliothis virescens* (Shelby, Adeyeye et al. 2000).

It has been suggested that melanization of self can be triggered in and by immune tissues alone, i.e. by hemocytes and the fat body (Avet-Rochex, Boyer et al. 2010): In a screen for melanization by knocking down 1340 genes in *Drosophila* melanization was exclusively observed in the fat body or hemocytes but not in non-immune tissues, i.e. imaginal discs, the glial cells, the central nervous system and the gut. The researchers however, did not investigate whether melanization was dependent on hemocytes.

Assuming that melanization of self requires encapsulation it becomes easy to understand why melanization is so rare. Steps that occur prior to melanization such as plasmatocyte infiltration, formation of septate junctions and lamellocytes infiltration take days to be completed. The development time of larvae comprises only approx. 4 days (at 25˚C) and is even shorter at 29˚C (the temperature where the experiments are carried out). It is hence conceivable that larvae simply run out of time to complete encapsulation before the onset of pupation.

Crystal cells are apparently the source of PPO during encapsulation of self since aberrant salivary glands never melanized in $Bc^{1}$ larvae despite the presence of lamellocytes that can also express PPO.

However, the signals that trigger PO-dependent melanization during self-encapsulation are unknown. The release of PPO from crystal cells requires Eiger and appears to be JNK dependent but is otherwise poorly understood. It has been

suggested that Eiger is secreted by the fat body during infection (Mabery and Schneider 2010). Possibly inner-membrane phospholipids such as PS exposed or released by aberrant tissue have a role in encapsulation-associated melanization.

Encapsulation in insects is most similar to granuloma formation in mammals (Hogan, Weinstock et al. 1999). Granulomas consist of macrophages that, similar to plasmatocytes in *Drosophila*, form a tight capsule around the pathogen (Adams 1976). Macrophages can fuse to form multinucleated giant cells (Spector 1976). In addition to macrophages, granulomas can also contain other cells including lymphocytes, neutrophils, fibroblasts and eosinophils. They can also contain ECM components such as collagen.

It should be noted that physicians also use the term granuloma to describe a small nodule, which in turn can be anything from a birthmark to a malignant tumor.

Pathological encapsulation-like granulomas are associated with various infectious diseases. Interestingly, they have also been linked to autoimmune diseases such as Wegener's granulomatosis (Kuntz, Beneke et al. 1967), Rheumatoid arthritis (Hessian, Highton et al. 2003), Crohn's disease (McGillis and Huntley 1989) and Churg–Strauss syndrome (Noth, Strek et al. 2003). Although, granuloma formation in cancer seems rare, macrophage infiltration of human tumors is a well-known phenomenon (Pages, Galon et al. 2010).

### 5.2.6 Does aberrant tissue inhibit lamellocyte proliferation *in trans*?

Surprisingly, in larvae with highest $Ras^{V12}$ expression (i.e. when crosses were kept at the optimal temperature for GAL4 activity) lamellocyte counts were lower than in larvae with little or no induction of $Ras^{V12}$ expression. In fact, lamellocyte numbers were highest in bleeds of parental control larvae that contain the *UAS-*$Ras^{V12}$ transgene only.

It is conceivable that plasmatocytes differentiate at the expense of lamellocytes since overproliferation of hemocytes was observed in larvae with $Ras^{V12}$ aberrant tissue compared to parental lines.

Lamellocytes were also found in other responder lines. It turned out that this hemocyte subtype is frequently observed in larvae that carry UAS-transgenes. However, lamellocytes were absent in wt ($w^{1118}$) or $Bx^{MS1096}$ *GAL4* larvae.

The reason for occasional apparently spontaneous or genetic background dependent lamellocyte differentiation is unknown. Possibly, larvae that carry UAS-transgenes of proteins involved in lamellocyte proliferation are more prone to contain lamellocytes, which might differentiate due to leaky, non-GAL4 induced transcription of the transgene. This could explain the presence of lamellocytes in *UAS-Ras$^{V12}$* larvae as Ras-signaling is involved in lamellocyte differentiation. This finding can however not explain the occurrences of lamellocytes in *UAS-grim* and *UAS-GFP* larvae. Possibly, integration of the UAS-site itself provides an unknown signal for lamellocyte differentiation. The fact that only one out of two *UAS-GFP* lines contains lamellocytes excludes the GFP transgene itself as the reason for lamellocyte proliferation. Possibly, the integration site of the transgene is involved in regulation of lamellocyte development.

### 5.2.7 PO and its role in control of tumor growth

Endogenous elicitors such as lipids (this work and (Bidla, Hauling et al. 2009)) and basement membrane fragments (Altincicek, Linder et al. 2007) activate PPO in insects independent of microbial stimuli, contributing to crosslinking and melanization soon after wounding. Also vertebrate tyrosinases that are related to PO can be activated by components of the basement membrane (Hedley, Gawkrodger et al. 1996). Here, I have shown that PPO is activated in tumor-containing larvae in the absence of foreign elicitors and I provide evidence that it plays a role in tumor growth control.

Although vertebrates do not possess an equivalent of the PPO system, the cascade leading to PO activation shares similarities with blood clotting/ coagulation in vertebrates. Both rely on serine proteases that are kept under tight control to prevent systemic activation. Interestingly, mammalian tumors can activate thrombosis, i.e. coagulation inside blood vessels is well recognized (Cushman 2007; Mackman 2008) thus supporting the idea that clotting restricts aberrant tissue growth in *Drosophila* larvae.

Moreover, evidence from *in vitro* and *in vivo* studies suggest that tumor growth and metastasis are in fact partially governed by coagulation (Kakkar and Macbeth 2010). The exact mechanism by which tumors activate clotting and how the clotting cascade controls tumor growth are however, poorly understood. The *Drosophila* tumor model

that I established might be a useful tool to understand the relationship between coagulation and tumor development.

### 5.2.8 TNF (Eiger) restricts growth of $Ras^{V12}$ salivary glands

In mammals, TNF is implicated in apoptosis induction and activation of the inflammatory response. Initially, TNF was identified for its ability to induce necrosis in certain experimental tumors (Carswell, Old et al. 1975; Helson, Green et al. 1975). However, it is nowadays undisputed that TNF actions can have both pro- and anti tumor consequences (Balkwill 2009). TNF binds to TNF receptors (TNFR 1 and 2), which when activated can basically have two outcomes dependent on the cellular context: cell survival and inflammation or apoptosis. The default response to TNF signaling is cell survival and inflammation but apoptosis is executed if NF-kappaB signaling that naturally antagonizes apoptosis is impaired. Other members of the TNF family such as FAS ligand and TRAIL however induce more rapid apoptosis via their respective receptors.

In natural killer (NK) cells TNF is an important cytotoxic effector against some tumor cells (Kashii, Giorda et al. 1999).

In contrast TNF made by malignant tumor cells is considered tumor-supportive: TNF signaling in tumor cells or in the tumor microenvironment where it acts in an autocrine or paracrine manner has been suggested to contribute to the induction of the tumorigenesis-promoting M2 phenotype of macrophages (see 5.2.10 and (Hagemann, Wilson et al. 2006)). TNF has also been implicated in ECM remodeling since the cytokine can induce expression of MMPs.

In *Drosophila*, recent studies suggest that the only TNF-like protein in flies, Eiger is essential for induction of JNK-mediated apoptosis of tumor cells that are deficient for cell polarity genes *scrib* and *l(2)gl* in epithelial tissue (Igaki, Pastor-Pareja et al. 2009). In contrast to the tumor limiting role of Eiger in *scrib* and *l(2)gl* depleted tumors, another study showed that TNF in hemocytes promotes tumorigensis by inducing MMP1 expression in $Ras^{V12}$; $scrib^{-/-}$ clones (Cordero, Macagno et al. 2010). Here, I show that partial loss of *eiger* both enhances growth of $Ras^{V12}$ salivary glands and of hemocyte infiltration resulting in a higher frequency of melanotic encapsulation. Increased overgrowth compared to wt in an *eiger* mutant background might be

explained with Eiger´s function as an apoptosis inducer. Supporting this I have shown that $Ras^{V12}$ salivary gland cells express active caspases. Possibly, adhering hemocytes induce apoptosis via Eiger since caspase-expressing $Ras^{V12}$ aberrant cells and hemocytes correlated while in contrast genetically inducing apoptosis in salivary gland cells did not attract hemocytes. Also the morphogenesis of salivary glands does not involve apoptotic cell death (Myat and Andrew 2000) and partially caspase-dependent salivary gland degradation does not occur before 12 hours after puparium formation (Baehrecke 2003) and activated Ras expressing salivary glands do not undergo autophagy (Berry and Baehrecke 2007).

An increase in hemocyte infiltration in Eiger-signaling impaired larvae might be explained by the assumption that aberrant cells that do not undergo apoptosis continue to secrete signals that attract hemocytes. The nature of these signals remains to be elucidated.

In conclusion, a role of TNF/ Eiger in tumor development appears to be conserved from flies to mammals and moreover seems universal for different tumors and tumor-like tissue. Like in mammals, the effect of TNF on these aberrant tissues seems paradox and might depend on the respective nature of the tissue. While TNF apparently promotes growth of tumors consisting of $Ras^{V12}$; *scrib*$^{-/-}$ epithelial clones it restricts growth of *scrib*$^{-/-}$ epithelial tumors and $Ras^{V12}$ salivary glands.

### 5.2.9 Eiger and the activation of PPO

It has previously been shown that Eiger is required for crystal cell activation, which is essential for PPO release in *Drosophila* larvae (Bidla, Dushay et al. 2007). Supposedly due to defects in crystal cell rupture, *eiger* mutants show reduced clot melanization as well as impaired activation of PPO, measured as melanization over time in a photometric assay. The observation that $Ras^{V12}$ salivary glands grew larger in an *egr*$^{3}$ mutant background compared to wt could hence also indicate that PO is effective against aberrant tissue, which would also be in line with the fact that the overgrowth of $Ras^{V12}$ salivary glands is enhanced in *Bc*$^{1}$ mutants. There are mutants available for genes whose function is better understood in regard to PPO activation. MP1 and MP2 are serine proteases that act upstream of PPO and appear to be essential for melanisation (Leclerc, Pelte et al. 2006; Tang, Kambris et al. 2006) MP1 and MP2

mutants have melanization defects and overexpression of these proteases in hemocytes leads to systemic melanization in larvae. Hence, MP1 and MP2 seem promising candidates to assess the role of PO in growth restriction of aberrant tissue.

### 5.2.10 A macrophages transition can be modeled in *Drosophila*

It is well recognized in vertebrates that macrophages infiltrate tumors where they either antagonize or promote tumor growth.

Analogous to T helper (Th) 1 and Th 2 cells, vertebrate macrophages can be classified into M1 and M2 type macrophages (or tumor-associated macrophages, TAMs). M1 macrophages are considered potent effector cells, which kill pathogens and destruct tumor cells by secreting cytotoxic molecules such as ROS and NO (Sica, Larghi et al. 2008). In contrast, M2 macrophages have scavenger function and are involved in tissue repair and remodeling. They have been shown to promote tumor growth by expressing ECM degrading enzymes such as MMPs (Allavena, Sica et al. 2008).

In this work, I show that plasmatocytes can be triggered to produce MMP solely by endogenous factors. The exact cause of MMP expression in $Bc^{1}$ mutant larvae that contain melanotic pre-activated crystal cells is unclear. However, it has been shown that melanization activates Drosomycin expression via the Toll pathway (Tang, Kambris et al. 2008) and $Tl^{8}$ mutants in which Toll is constitutively active also express MMP in hemocytes (Irving, Ubeda et al. 2005). Moreover in humans, MMP9 expression can be triggered via Toll-like receptors in monocytes and macrophages (Gebbia, Coleman et al. 2004; Elass, Aubry et al. 2005).

Products of the PPO cascade might hence indirectly also cause upregulation of MMP in plasmatocytes that in turn leads to increased overgrowth infiltrated tissue.

In vertebrates, MMPs are upregulated in the presence NO, ROS and oxidized lipoproteins (which play a role in thrombosis) while scavenging ROS and medically lowering lipid content in patients decreased MMP expression in macrophages (Galis and Khatri 2002). Whether the same factors play a role in MMP regulation in *Drosophila* remains to be investigated.

### 5.2.11 Potential immune-stimulator signals released by aberrant tissue

Cytokines of the unpaired family supposedly affect hemocyte proliferation (Pastor-Pareja, Wu et al. 2008). ROS such as hydrogen peroxide have been shown to be involved in hemocyte recruitment to wound sites in *Drosophila* embryos and in zebrafish (Niethammer, Grabher et al. 2009; Moreira, Stramer et al. 2010). Furthermore, it has been reported in *Drosophila* that DNA, which has escaped apoptotic degradation can trigger a humoral immune response, i.e. fat body expression of Diptericin and Attacin (Mukae, Yokoyama et al. 2002). Nucleic acids have also been shown to activate immunity in other insects (Altincicek, Stotzel et al. 2008) and in vertebrates where mitochondrial DNA can trigger an inflammatory response (Zhang, Raoof et al. 2010). Moreover, ECM fragments can induce apoptosis in mammalian endothelial cells (Cheresh and Stupack 2008). It is hence tempting to speculate that MMPs, secreted by $Ras^{V12}$ salivary glands are causing the apoptotic phenotype of the tissue in a paracrine or autocrine manner.

### 5.2.12 Wound healing and cancer

Tumors have often been described as wounds that never heal. Hence, in larvae with tumor-like tissue overgrowth, a response similar to that in wound healing processes would be expected.

Wound healing in *Drosophila* larvae comprises coagulation including PO activity, leading to clot hardening and eventually melanization at the wound site. In both embryos and larvae, hemocytes also temporarily migrate to the epithelial wound sites to participate in the repair process (Galko and Krasnow 2004; Stramer, Wood et al. 2005; Babcock, Brock et al. 2008).

It has furthermore been shown that hemocytes migrate to both neoplastic and mechanically damaged imaginal discs thereby activating JAK/STAT signaling in the fat body (Pastor-Pareja, Wu et al. 2008). By showing that hemocyte migration also occurs as a response to aberrant polyploid tissues such as fat body and salivary glands I have demonstrated that hemocyte migration towards wounds is independent of the mitotic nature of the tissue.

While in embryos several signals that control hemocyte motility have been described (Wood, Faria et al. 2006) the underlying stimuli that guide larval hemocytes to wound

sites are much less understood and clearly different from the chemo-attractants that are responsible for mobility of embryonic hemocytes (Babcock, Brock et al. 2008). According to Matzinger, patrolling immune cells promiscuously recognize hydrophobic surfaces as exposed on dying cells rather then (or in addition to) specific microbial patterns (Matzinger 2007). Consistent with this idea and with data showing that larval hemocytes adhere to cell debris at wound sites (Babcock, Brock et al. 2008), I have shown in this work that hemocytes bind to partially necrotic cells.

### 5.2.13 Implications for understanding human cancer

The association between cancer and the immune system has been recognized already late in the 19$^{th}$ century by the German pathologist Rudolph Virchow. Clinical studies support both anti- and pro-tumor roles of the immune system but the mechanisms, which decide whether immune reactions against tumors have a positive or negative outcome for the patient are largely unknown. I have shown that *Drosophila* tumor-like salivary glands, which express one of the most common oncogene in human cancers - activated Ras - are affected by TNF signaling and are infiltrated by macrophage-like cells that supposedly have tumor growth supporting and antagonizing functions reminiscent of M1 and M2 macrophages in tumor microenvironments in humans. I envision that this tumor model can substantially contribute to the understanding of interactions between innate immunity/ inflammation and tumor development.

### 5.2.14 Ongoing experiments

I have presented strong evidence that aberrant, tumor-like salivary glands partially undergo apoptosis since some gland cells contain active caspases and their DNA is fragmented. Cells might however also undergo necrosis and factors related to these two types of cell death might in turn have an immune-stimulatory role. Coexpressing apoptosis inhibitors such as viral p35 will allow evaluating the contribution of necrosis on tumor death and its role in immune-activation. Apoptosis induction via Eiger-Wengen requires the endocytotic pathway (Igaki, Pastor-Pareja et al. 2009). Endocytosis can effectively be compromised by expressing dominant-negative Rab5

(Rab5$^{DN}$). I have preliminary results indicating that co-expression of Rab5$^{DN}$ with an *l(2)gl* knockdown construct under *Bx$^{ms1096}$ GAL4* strongly enhances aberrant growth in wing imaginal discs and results in giant larvae that either die as dauer-stage larvae or at the onset of pupation, basically phenocopying homozygous *l(2)gl* loss-of-function larvae. This demonstrates that interference with endocytosis in tumor cells indeed prevents cell death and is in line with earlier reports (Igaki, Pastor-Pareja et al. 2009).

In addition, I observed melanotic encapsulation of salivary glands in some of these larvae, suggesting that encapsulation is the general defense mechanism against larger-celled tumors irrespective of the genetic nature of that tumor, i.e. encapsulation is not specific for dominant-active Ras expressing tumors.

To investigate if properties related to the metastatic potential of the tumor shift the cellular immune response from hemocyte infiltration to encapsulation I coexpressed *Ras$^{V12}$* and a *scrib*$^{RNAi}$ construct since the combination of activated Ras with *scrib* depletion has previously been shown to lead to extreme overgrowth phenotypes in eye disc tumors. *UAS-scrib$^{RNAi}$* ; *UAS-Ras$^{V12}$* under the control of *Bx$^{ms1096}$ GAL4* seems to increase the frequency of melanization of salivary glands and also leads to more severe overgrowth of wing imaginal discs compared to Ras$^{V12}$ alone.

Based on my findings I propose a model by which overgrowth of aberrant salivary glands and hemocyte infiltration is regulated: Induction of Ras$^{V12}$ in salivary glands triggers MMP expression, which in turn catalyses the breakdown of the surrounding ECM. ECM fragments serve as immune elicitors as well as Eiger. Hemocytes adhere to the tissue to ultimately encapsulate the tissue. Lamellocytes and crystal cells are putatively attracted to the aberrant tissue by plasmatocyte-derived signals since they adhere only in conjunction with plasmatocytes. Survival of the aberrant gland enhances encapsulation. PO, phagocytosis and Eiger-Wengen signaling might restrict aberrant growth, while hemocyte-provided MMP might enhance the growth of aberrant salivary glands (see Figure 31).

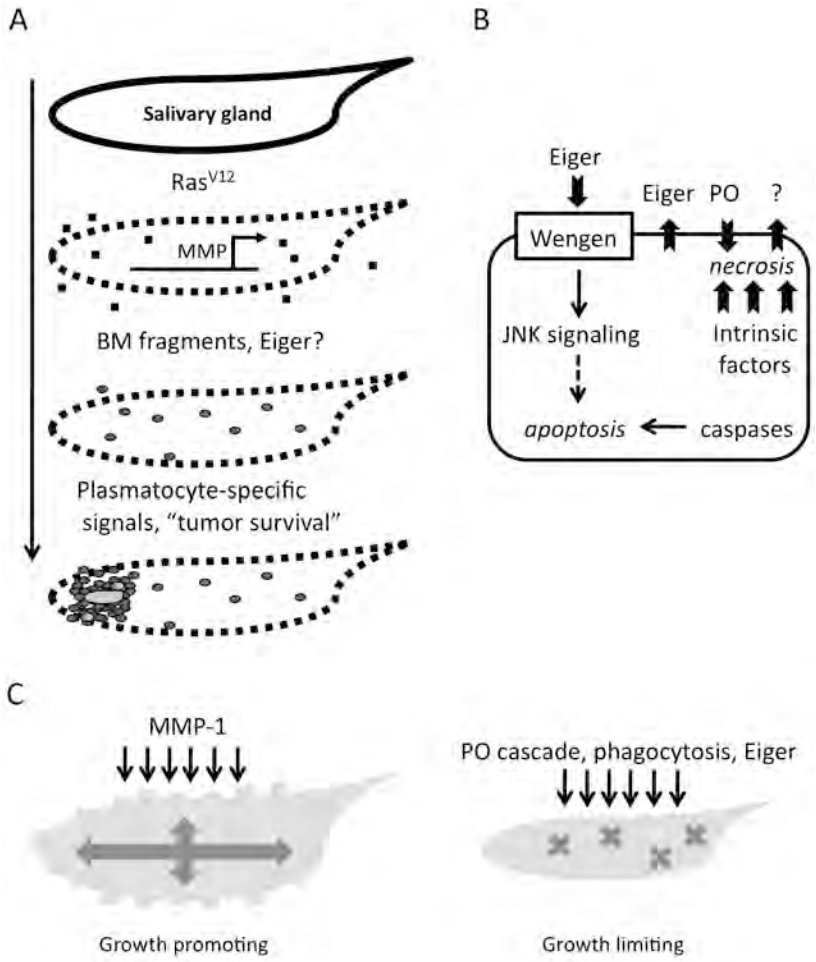


**Figure 31. Tumor-like growth and immune response.** (A) Steps of salivary gland encapsulation. Ras$^{V12}$ expression induces MMP expression, which in turn leads to degradation of basement membrane (BM, black line). Diffusing BM fragments (black squares) attract hemocytes to the tumor and also Eiger might activate the immune system. Plasmatocytes (small grey circles) attach to the gland, spread and form a layer. Onto this layer crystal cells (larger circles) and lamellocytes (grey) adhere and form a capsule, which can melanize in a crystal cell dependant fashion. Sustained blocking of apoptosis via *wengen* knockdown enhances encapsulation. (B) Interaction between immune effectors and tumor cells. Eiger present in the hemolymph binds its receptor Wengen on the tumor and induces apoptosis. Other extrinsic and intrinsic effectors (PO?) restrict tumor growth by inducing necrotic cell death. (C) Tumor growth promoting and limiting activities of the immune system. Extrinsic MMP-1, expressed by plasmatocytes might support gland-borne MMP in BM degradation. This in turn aids gland growth. The PPO cascade, phagocytosis and Eiger-induced apoptosis putatively restrict aberrant growth.

# 6 Conclusion and outlook

This work addresses the question essential for immunology in general and autoimmunity, including cancer in particular: How is the immune system activated in the absence of infection, i.e. in the absence of foreign, non-self elicitors?

I have shown that aberrant apoptosis can activate melanization, putatively due to exposure of hydrophobic phospholipids, which might provide an assembly platform for the PPO-activating complex analogous to platelet activation in mammals. Altered self was also induced by expressing oncogenic, i.e. dominant-activate Ras and by knocking down tumor suppressor genes resulting in a loss of tissue architecture and tissue overgrowth. Such aberrant tissue induced a complex immune response involving both humoral and cellular effectors ultimately resulting in encapsulation. Possibly similar to TNF in mammals, dTNF/ Eiger signaling appears to contribute to the death of aberrant cells. However, the source of Eiger is not clear. It is conceivable that adhering hemocytes secrete Eiger and thereby induce apoptosis. Alternatively or in addition, Eiger might be secreted by $Ras^{V12}$ aberrant tissue and act in a paracrine or autocrine manner.

In addition, I have shown that tissue-infiltrating immune cells can be induced to express MMP, reminiscent of tumor-associated macrophages in humans. The underlying mechanisms that trigger the transition from naïve hemocytes to MMP-expressing hemocytes, which putatively support aberrant overgrowth can be investigated with the model presented in this work.

Auto-encapsulation of immune tissue has been described in *Drosophila* mutants. However, for the first time I show that tumor-like overgrowth of non-immune tissue can induce encapsulation.

Another novel finding was that PO, which is important in wound healing and coagulation appears to restrict the growth of such tissue. The mutant $Bc^{1}$ that lacks functional crystal cells and hence PO appears to have additional immune defects. Further experiments to proof the involvement of PO in tumor control need to be done. Two proteases that activate PPO have been described: MP1 and MP2, both of which are available as loss-of-function mutants. Tumor induction in respective mutants will help elucidating the role of PO in the defense against tumors. Likewise,

immune mutants that likely have an impact on tumorigenesis can be tested, namely mutants of TEPs and of genes involved in clotting/ coagulation since clotting and PPO activation are linked and coagulation is involved in human tumorigenesis.
Given the similarities between the immune response against mammalian tumors and tumor-like tissue in *Drosophila* (i.e. macrophage invasion, involvement of TNF / Eiger signaling and MMP expression) my findings might help to establish *Drosophila* as a model to study tumor – immune interactions.

# 7 Acknowledgements

At this point I want to thank a number of people for their support, discussions and other contributions:
Foremost, my supervisor Uli Theopold for inspiring discussions & for giving me a lot of freedom in designing and carrying out my project.
Danke Wolfgang Nellen, dass du mir die Promotion an der Universität Kassel ermöglicht hast.
Zhi – my dear friend and wonderful colleague. I want to thank you for great company, discussions and support, not to forget the countless vials that I "borrowed" from you!
Tine - my "fadder" - for introducing me to the art of fly pushing, and for your great organization skills. Gawa, Malin, Christine and Robert – Thanks for method introductions and discussions. Michaela and Ken – you have been dedicated lab students. The "Pavels": Thank you for support and interesting discussions in and outside of the lab.
The "innate immunity people" at SU for reagents and nice moments.
People at the Department of Molecular Biology & Functional Genomics – I like to highlight Gunnel Björklund for maintenance of the fly room and Britta Tumlin: Tusen tack för all flygmaten!
Samakovlis lab: Thanks for all the advice, spared aliquots and stuff!
The Department of Zoology for sharing their microscope.
Thorsten, Kristian, Micke and Deike, Roger, Anders and Shawon - thank you for discussions and for being supportive friends!

This work could not have been completed without the financial support from the German Academic Exchange Service (DAAD) and the Royal Swedish Academy of Science (KVA). Danke and Tack!

Lastly, it would have been impossible to pursue this project without the support & encouragement of my parents and my brother whom I can rely on at all times!

# 8 References


Adams, D. O. (1976). "The granulomatous inflammatory response. A review." Am J Pathol **84**(1): 164-192.

Adams, J. M. and S. Cory (2007). "The Bcl-2 apoptotic switch in cancer development and therapy." Oncogene **26**(9): 1324-1337.

Agaisse, H., U. M. Petersen, et al. (2003). "Signaling role of hemocytes in Drosophila JAK/STAT-dependent response to septic injury." Dev Cell **5**(3): 441-450.

Aggarwal, K. and N. Silverman (2008). "Positive and negative regulation of the Drosophila immune response." BMB Rep **41**(4): 267-277.

Allavena, P., A. Sica, et al. (2008). "The Yin-Yang of tumor-associated macrophages in neoplastic progression and immune surveillance." Immunol Rev **222**: 155-161.

Altincicek, B., M. Linder, et al. (2007). "Microbial metalloproteinases mediate sensing of invading pathogens and activate innate immune responses in the lepidopteran model host Galleria mellonella." Infect Immun **75**(1): 175-183.

Altincicek, B., S. Stotzel, et al. (2008). "Host-derived extracellular nucleic acids enhance innate immune responses, induce coagulation, and prolong survival upon infection in insects." J Immunol **181**(4): 2705-2712.

Altincicek, B. and A. Vilcinskas (2006). "Metamorphosis and collagen-IV-fragments stimulate innate immune response in the greater wax moth, Galleria mellonella." Dev Comp Immunol **30**(12): 1108-1118.

Altincicek, B. and A. Vilcinskas (2008). "Identification of a lepidopteran matrix metalloproteinase with dual roles in metamorphosis and innate immunity." Dev Comp Immunol **32**(4): 400-409.

Avet-Rochex, A., K. Boyer, et al. (2010). "An in vivo RNA interference screen identifies gene networks controlling Drosophila melanogaster blood cell homeostasis." BMC Dev Biol **10**: 65.

Ayres, J. S. and D. S. Schneider (2008). "A signaling protease required for melanization in Drosophila affects resistance and tolerance of infections." PLoS Biol **6**(12): 2764-2773.

Babcock, D. T., A. R. Brock, et al. (2008). "Circulating blood cells function as a surveillance system for damaged tissue in Drosophila larvae." Proc Natl Acad Sci U S A **105**(29): 10017-10022.

Baehrecke, E. H. (2003). "Autophagic programmed cell death in Drosophila." Cell Death Differ **10**(9): 940-945.

Bahri, S., S. Wang, et al. (2010). "The leading edge during dorsal closure as a model for epithelial plasticity: Pak is required for recruitment of the Scribble complex and septate junction formation." Development **137**(12): 2023-2032.

Balkwill, F. (2009). "Tumour necrosis factor and cancer." Nat Rev Cancer **9**(5): 361-371.

Barry, O. P. and G. A. FitzGerald (1999). "Mechanisms of cellular activation by platelet microparticles." Thromb Haemost **82**(2): 794-800.

Berry, D. L. and E. H. Baehrecke (2007). "Growth arrest and autophagy are required for salivary gland cell degradation in Drosophila." Cell **131**(6): 1137-1148.

Bidla, G., M. S. Dushay, et al. (2007). "Crystal cell rupture after injury in Drosophila requires the JNK pathway, small GTPases and the TNF homolog Eiger." J Cell Sci **120**(Pt 7): 1209-1215.

Bidla, G., T. Hauling, et al. (2009). "Activation of insect phenoloxidase after injury: endogenous versus foreign elicitors." J Innate Immun **1**(4): 301-308.

Bidla, G., M. Lindgren, et al. (2005). "Hemolymph coagulation and phenoloxidase in Drosophila larvae." Dev Comp Immunol **29**(8): 669-679.

Blandin, S. and E. A. Levashina (2004). "Thioester-containing proteins and insect immunity." Mol Immunol **40**(12): 903-908.

Blandin, S., S. H. Shiao, et al. (2004). "Complement-like protein TEP1 is a determinant of vectorial capacity in the malaria vector Anopheles gambiae." Cell **116**(5): 661-670.

Boman, H. G. and H. Steiner (1981). "Humoral immunity in Cecropia pupae." Curr Top Microbiol Immunol **94-95**: 75-91.

Bosch, M., F. Serras, et al. (2005). "JNK signaling pathway required for wound healing in regenerating Drosophila wing imaginal discs." Dev Biol **280**(1): 73-86.

Bou Aoun, R., C. Hetru, et al. (2011). "Analysis of thioester-containing proteins during the innate immune response of Drosophila melanogaster." J Innate Immun **3**(1): 52-64.

Boutros, M., H. Agaisse, et al. (2002). "Sequential activation of signaling pathways during innate immune responses in Drosophila." Dev Cell **3**(5): 711-722.

Braun, A., J. A. Hoffmann, et al. (1998). "Analysis of the Drosophila host defense in domino mutant larvae, which are devoid of hemocytes." Proc Natl Acad Sci U S A **95**(24): 14337-14342.

Brumby, A. M. and H. E. Richardson (2003). "scribble mutants cooperate with oncogenic Ras or Notch to cause neoplastic overgrowth in Drosophila." EMBO J **22**(21): 5769-5779.

Brumby, A. M. and H. E. Richardson (2005). "Using Drosophila melanogaster to map human cancer pathways." Nat Rev Cancer **5**(8): 626-639.

Bulet, P., C. Hetru, et al. (1999). "Antimicrobial peptides in insects; structure and function." Dev Comp Immunol **23**(4-5): 329-344.

Burnet, F. M. (1970). "The concept of immunological surveillance." Prog Exp Tumor Res **13**: 1-27.

Burnet, M. (1957). "Cancer; a biological approach. I. The processes of control." Br Med J **1**(5022): 779-786.

Carswell, E. A., L. J. Old, et al. (1975). "An endotoxin-induced serum factor that causes necrosis of tumors." Proc Natl Acad Sci U S A **72**(9): 3666-3670.

Castillo, J. C., A. E. Robertson, et al. (2006). "Characterization of hemocytes from the mosquitoes Anopheles gambiae and Aedes aegypti." Insect Biochem Mol Biol **36**(12): 891-903.

Cerenius, L., B. L. Lee, et al. (2008). "The proPO-system: pros and cons for its role in invertebrate immunity." Trends Immunol **29**(6): 263-271.

Cerenius, L. and K. Soderhall (2004). "The prophenoloxidase-activating system in invertebrates." Immunol Rev **198**: 116-126.

Chen, G. and D. V. Goeddel (2002). "TNF-R1 signaling: a beautiful pathway." Science **296**(5573): 1634-1635.

Cheresh, D. A. and D. G. Stupack (2008). "Regulation of angiogenesis: apoptotic cues from the ECM." Oncogene **27**(48): 6285-6298.

Ciurana, C. L., B. Zwart, et al. (2004). "Complement activation by necrotic cells in normal plasma environment compares to that by late apoptotic cells and involves predominantly IgM." Eur J Immunol **34**(9): 2609-2619.

Clark, A. G., M. B. Eisen, et al. (2007). "Evolution of genes and genomes on the Drosophila phylogeny." Nature **450**(7167): 203-218.

Colinet, D., A. Dubuffet, et al. (2009). "A serpin from the parasitoid wasp Leptopilina boulardi targets the Drosophila phenoloxidase cascade." Dev Comp Immunol **33**(5): 681-689.

Cordero, J. B., J. P. Macagno, et al. (2010). "Oncogenic Ras diverts a host TNF tumor suppressor activity into tumor promoter." Dev Cell **18**(6): 999-1011.

Crozatier, M. and M. Meister (2007). "Drosophila haematopoiesis." Cell Microbiol **9**(5): 1117-1126.

Cushman, M. (2007). "Epidemiology and risk factors for venous thrombosis." Semin Hematol **44**(2): 62-69.

De Gregorio, E., S. J. Han, et al. (2002). "An immune-responsive Serpin regulates the melanization cascade in Drosophila." Dev Cell **3**(4): 581-592.

De Gregorio, E., P. T. Spellman, et al. (2002). "The Toll and Imd pathways are the major regulators of the immune response in Drosophila." EMBO J **21**(11): 2568-2579.

Deane, J. A. and S. Bolland (2006). "Nucleic acid-sensing TLRs as modifiers of autoimmunity." J Immunol **177**(10): 6573-6578.

Delaney, J. R., S. Stoven, et al. (2006). "Cooperative control of Drosophila immune responses by the JNK and NF-kappaB signaling pathways." EMBO J **25**(13): 3068-3077.

Dietzl, G., D. Chen, et al. (2007). "A genome-wide transgenic RNAi library for conditional gene inactivation in Drosophila." Nature **448**(7150): 151-156.

Dostert, C., E. Jouanguy, et al. (2005). "The Jak-STAT signaling pathway is required but not sufficient for the antiviral response of drosophila." Nat Immunol **6**(9): 946-953.

Dow, L. E., A. M. Brumby, et al. (2003). "hScrib is a functional homologue of the Drosophila tumour suppressor Scribble." Oncogene **22**(58): 9225-9230.

Dunn, G. P., A. T. Bruce, et al. (2002). "Cancer immunoediting: from immunosurveillance to tumor escape." Nat Immunol **3**(11): 991-998.

Ehrchen, J. M., C. Sunderkotter, et al. (2009). "The endogenous Toll-like receptor 4 agonist S100A8/S100A9 (calprotectin) as innate amplifier of infection, autoimmunity, and cancer." J Leukoc Biol **86**(3): 557-566.

Elass, E., L. Aubry, et al. (2005). "Mycobacterial lipomannan induces matrix metalloproteinase-9 expression in human macrophagic cells through a Toll-like receptor 1 (TLR1)/TLR2- and CD14-dependent mechanism." Infect Immun **73**(10): 7064-7068.

Elliott, M. R. and K. S. Ravichandran (2008). "Death in the CNS: six-microns-under." Cell **133**(3): 393-395.

Erturk-Hasdemir, D. and N. Silverman (2005). "Eater: a big bite into phagocytosis." Cell **123**(2): 190-192.

Eslin, P. and G. Doury (2006). "The fly Drosophila subobscura: a natural case of innate immunity deficiency." Dev Comp Immunol **30**(11): 977-983.

Evan, G. and T. Littlewood (1998). "A matter of life and cell death." Science **281**(5381): 1317-1322.

Ferrandon, D., J. L. Imler, et al. (2007). "The Drosophila systemic immune response: sensing and signalling during bacterial and fungal infections." Nat Rev Immunol **7**(11): 862-874.

Ferrandon, D., A. C. Jung, et al. (1998). "A drosomycin-GFP reporter transgene reveals a local immune response in Drosophila that is not dependent on the Toll pathway." EMBO J **17**(5): 1217-1227.

Franc, N. C., P. Heitzler, et al. (1999). "Requirement for croquemort in phagocytosis of apoptotic cells in Drosophila." Science **284**(5422): 1991-1994.

Gaipl, U. S., S. Kuenkele, et al. (2001). "Complement binding is an early feature of necrotic and a rather late event during apoptotic cell death." Cell Death Differ **8**(4): 327-334.

Galis, Z. S. and J. J. Khatri (2002). "Matrix metalloproteinases in vascular remodeling and atherogenesis: the good, the bad, and the ugly." Circ Res **90**(3): 251-262.

Galko, M. J. and M. A. Krasnow (2004). "Cellular and genetic analysis of wound healing in Drosophila larvae." PLoS Biol **2**(8): E239.

Gateff, E. (1978). "Malignant neoplasms of genetic origin in Drosophila melanogaster." Science **200**(4349): 1448-1459.

Gateff, E. and H. A. Schneiderman (1969). "Neoplasms in mutant and cultured wild-tupe tissues of Drosophila." Natl Cancer Inst Monogr **31**: 365-397.

Gebbia, J. A., J. L. Coleman, et al. (2004). "Selective induction of matrix metalloproteinases by Borrelia burgdorferi via toll-like receptor 2 in monocytes." J Infect Dis **189**(1): 113-119.

Gluderer, S., S. Oldham, et al. (2008). "Bunched, the Drosophila homolog of the mammalian tumor suppressor TSC-22, promotes cellular growth." BMC Dev Biol **8**: 10.

Greenspan, R. J. (1997). Fly pushing : the theory and practice of Drosophila genetics. Cold Spring Harbor, NY, Cold Spring Harbor Laboratory Press.

Grifoni, D., F. Garoia, et al. (2004). "The human protein Hugl-1 substitutes for Drosophila lethal giant larvae tumour suppressor function in vivo." Oncogene **23**(53): 8688-8694.

Grimsley, C. and K. S. Ravichandran (2003). "Cues for apoptotic cell engulfment: eat-me, don't eat-me and come-get-me signals." Trends Cell Biol **13**(12): 648-656.

Ha, E. M., C. T. Oh, et al. (2005). "A direct role for dual oxidase in Drosophila gut immunity." Science **310**(5749): 847-850.

Ha, E. M., C. T. Oh, et al. (2005). "An antioxidant system required for host protection against gut infection in Drosophila." Dev Cell **8**(1): 125-132.

Hagemann, T., J. Wilson, et al. (2006). "Ovarian cancer cells polarize macrophages toward a tumor-associated phenotype." J Immunol **176**(8): 5023-5032.

Halder, G. and R. L. Johnson (2011). "Hippo signaling: growth control and beyond." Development **138**(1): 9-22.

Hedley, S., D. J. Gawkrodger, et al. (1996). "Investigation of the influence of extracellular matrix proteins on normal human melanocyte morphology and melanogenic activity." Br J Dermatol **135**(6): 888-897.

Helson, L., S. Green, et al. (1975). "Effect of tumour necrosis factor on cultured human melanoma cells." Nature **258**(5537): 731-732.

Hessian, P. A., J. Highton, et al. (2003). "Cytokine profile of the rheumatoid nodule suggests that it is a Th1 granuloma." Arthritis Rheum **48**(2): 334-338.

Hogan, L. H., J. V. Weinstock, et al. (1999). "TCR specificity in infection induced granulomas." Immunol Lett **68**(1): 115-120.

Honti, V., G. Csordas, et al. (2010). "Cell lineage tracing reveals the plasticity of the hemocyte lineages and of the hematopoietic compartments in Drosophila melanogaster." Mol Immunol **47**(11-12): 1997-2004.

Horstman, L. L. and Y. S. Ahn (1999). "Platelet microparticles: a wide-angle perspective." Crit Rev Oncol Hematol **30**(2): 111-142.

Humbert, P., S. Russell, et al. (2003). "Dlg, Scribble and Lgl in cell polarity, cell proliferation and cancer." Bioessays **25**(6): 542-553.

Humbert, P. O., N. A. Grzeschik, et al. (2008). "Control of tumourigenesis by the Scribble/Dlg/Lgl polarity module." Oncogene **27**(55): 6888-6907.

Igaki, T., H. Kanda, et al. (2002). "Eiger, a TNF superfamily ligand that triggers the Drosophila JNK pathway." EMBO J **21**(12): 3009-3018.

Igaki, T., J. C. Pastor-Pareja, et al. (2009). "Intrinsic Tumor Suppression and Epithelial Maintenance by Endocytic Activation of Eiger/TNF Signaling in Drosophila." Developmental Cell **16**(3): 458-465.

Igaki, T., J. C. Pastor-Pareja, et al. (2009). "Intrinsic tumor suppression and epithelial maintenance by endocytic activation of Eiger/TNF signaling in Drosophila." Dev Cell **16**(3): 458-465.

Imler, J. L. and P. Bulet (2005). "Antimicrobial peptides in Drosophila: structures, activities and gene regulation." Chem Immunol Allergy **86**: 1-21.

Irving, P., L. Troxler, et al. (2001). "A genome-wide analysis of immune responses in Drosophila." Proc Natl Acad Sci U S A **98**(26): 15119-15124.

Irving, P., J. M. Ubeda, et al. (2005). "New insights into Drosophila larval haemocyte functions through genome-wide analysis." Cell Microbiol **7**(3): 335-350.

Jiang, H., P. H. Patel, et al. (2009). "Cytokine/Jak/Stat signaling mediates regeneration and homeostasis in the Drosophila midgut." Cell **137**(7): 1343-1355.

Jung, S. H., C. J. Evans, et al. (2005). "The Drosophila lymph gland as a developmental model of hematopoiesis." Development **132**(11): 2521-2533.

Kakkar, A. K. and F. Macbeth (2010). "Antithrombotic therapy and survival in patients with malignant disease." Br J Cancer **102 Suppl 1**: S24-29.

Kallio, J., A. Leinonen, et al. (2005). "Functional analysis of immune response genes in Drosophila identifies JNK pathway as a regulator of antimicrobial peptide gene expression in S2 cells." Microbes Infect **7**(5-6): 811-819.

Kanda, H., T. Igaki, et al. (2002). "Wengen, a member of the Drosophila tumor necrosis factor receptor superfamily, is required for Eiger signaling." J Biol Chem **277**(32): 28372-28375.

Karim, F. D. and G. M. Rubin (1998). "Ectopic expression of activated Ras1 induces hyperplastic growth and increased cell death in Drosophila imaginal tissues." Development **125**(1): 1-9.

Kashii, Y., R. Giorda, et al. (1999). "Constitutive expression and role of the TNF family ligands in apoptotic killing of tumor cells by human NK cells." J Immunol **163**(10): 5358-5366.

Kessenbrock, K., V. Plaks, et al. (2010). "Matrix metalloproteinases: regulators of the tumor microenvironment." Cell **141**(1): 52-67.

Kim, L. K., U. Y. Choi, et al. (2007). "Down-regulation of NF-kappaB target genes by the AP-1 and STAT complex during the innate immune response in Drosophila." PLoS Biol **5**(9): e238.

Kim, T., J. Yoon, et al. (2005). "Downregulation of lipopolysaccharide response in Drosophila by negative crosstalk between the AP1 and NF-kappaB signaling modules." Nat Immunol **6**(2): 211-218.

Kleino, A., S. Valanne, et al. (2005). "Inhibitor of apoptosis 2 and TAK1-binding protein are components of the Drosophila Imd pathway." EMBO J **24**(19): 3423-3434.

Kocks, C., J. H. Cho, et al. (2005). "Eater, a transmembrane protein mediating phagocytosis of bacterial pathogens in Drosophila." Cell **123**(2): 335-346.

Korpos, E., C. Wu, et al. (2010). "Role of the extracellular matrix in lymphocyte migration." Cell Tissue Res **339**(1): 47-57.

Kuntz, E., G. Beneke, et al. (1967). "[Wegener's granulomatosis]." Med Welt **6**: 295-304 passim.

Kurada, P. and K. White (1998). "Ras promotes cell survival in Drosophila by downregulating hid expression." Cell **95**(3): 319-329.

Kurata, S. (2010). "Extracellular and intracellular pathogen recognition by Drosophila PGRP-LE and PGRP-LC." Int Immunol **22**(3): 143-148.

Kurucz, E., B. Vaczi, et al. (2007). "Definition of Drosophila hemocyte subsets by cell-type specific antigens." Acta Biol Hung **58 Suppl**: 95-111.

Kurucz, E., C. J. Zettervall, et al. (2003). "Hemese, a hemocyte-specific transmembrane protein, affects the cellular immune response in Drosophila." Proc Natl Acad Sci U S A **100**(5): 2622-2627.

Lagueux, M., E. Perrodou, et al. (2000). "Constitutive expression of a complement-like protein in toll and JAK gain-of-function mutants of Drosophila." Proc Natl Acad Sci U S A **97**(21): 11427-11432.

Lavine, M. D. and M. R. Strand (2002). "Insect hemocytes and their role in immunity." Insect Biochem Mol Biol **32**(10): 1295-1309.

Leclerc, V., N. Pelte, et al. (2006). "Prophenoloxidase activation is not required for survival to microbial infections in Drosophila." EMBO Rep **7**(2): 231-235.

Lemaitre, B. and J. Hoffmann (2007). "The host defense of Drosophila melanogaster." Annu Rev Immunol **25**: 697-743.

Lesch, C., A. Goto, et al. (2007). "A role for Hemolectin in coagulation and immunity in Drosophila melanogaster." Dev Comp Immunol **31**(12): 1255-1263.

Ligoxygakis, P., N. Pelte, et al. (2002). "A serpin mutant links Toll activation to melanization in the host defence of Drosophila." EMBO J **21**(23): 6330-6337.

Lindgren, M., R. Riazi, et al. (2008). "Fondue and transglutaminase in the Drosophila larval clot." J Insect Physiol **54**(3): 586-592.

Llano, E., G. Adam, et al. (2002). "Structural and enzymatic characterization of Drosophila Dm2-MMP, a membrane-bound matrix metalloproteinase with tissue-specific expression." J Biol Chem **277**(26): 23321-23329.

Lue, R. A., S. M. Marfatia, et al. (1994). "Cloning and characterization of hdlg: the human homologue of the Drosophila discs large tumor suppressor binds to protein 4.1." Proc Natl Acad Sci U S A **91**(21): 9818-9822.

Mabery, E. M. and D. S. Schneider (2010). "The Drosophila TNF ortholog eiger is required in the fat body for a robust immune response." J Innate Immun **2**(4): 371-378.

Mackman, N. (2008). "Triggers, targets and treatments for thrombosis." Nature **451**(7181): 914-918.

Manaka, J., T. Kuraishi, et al. (2004). "Draper-mediated and phosphatidylserine-independent phagocytosis of apoptotic cells by Drosophila hemocytes/macrophages." J Biol Chem **279**(46): 48466-48476.

Mantovani, A. and A. Sica (2010). "Macrophages, innate immunity and cancer: balance, tolerance, and diversity." Curr Opin Immunol **22**(2): 231-237.

Markus, R., E. Kurucz, et al. (2005). "Sterile wounding is a minimal and sufficient trigger for a cellular immune response in Drosophila melanogaster." Immunol Lett **101**(1): 108-111.

Markus, R., B. Laurinyecz, et al. (2009). "Sessile hemocytes as a hematopoietic compartment in Drosophila melanogaster." Proc Natl Acad Sci U S A **106**(12): 4805-4809.

Matzinger, P. (2002). "The danger model: a renewed sense of self." Science **296**(5566): 301-305.

Matzinger, P. (2007). "Friendly and dangerous signals: is the tissue in control?" Nat Immunol **8**(1): 11-13.

McGillis, S. T. and A. C. Huntley (1989). "Metastatic Crohn's disease." West J Med **151**(2): 203-205.

McGuire, S. E., P. T. Le, et al. (2003). "Spatiotemporal rescue of memory dysfunction in Drosophila." Science **302**(5651): 1765-1768.

Meister, M. (2004). "Blood cells of Drosophila: cell lineages and role in host defence." Curr Opin Immunol **16**(1): 10-15.

Meister, M. and M. Lagueux (2003). "Drosophila blood cells." Cell Microbiol **5**(9): 573-580.

Meister, M., B. Lemaitre, et al. (1997). "Antimicrobial peptide defense in Drosophila." Bioessays **19**(11): 1019-1026.

Menendez, J., A. Perez-Garijo, et al. (2010). "A tumor-suppressing mechanism in Drosophila involving cell competition and the Hippo pathway." Proc Natl Acad Sci U S A **107**(33): 14651-14656.

Milchanowski, A. B., A. L. Henkenius, et al. (2004). "Identification and characterization of genes involved in embryonic crystal cell formation during Drosophila hematopoiesis." Genetics **168**(1): 325-339.

Minakhina, S. and R. Steward (2006). "Melanotic mutants in Drosophila: pathways and phenotypes." Genetics **174**(1): 253-263.

Moffat, K. G., J. H. Gould, et al. (1992). "Inducible cell ablation in Drosophila by cold-sensitive ricin A chain." Development **114**(3): 681-687.

Molina-Cruz, A., R. J. DeJong, et al. (2008). "Reactive oxygen species modulate Anopheles gambiae immunity against bacteria and Plasmodium." J Biol Chem **283**(6): 3217-3223.

Moreau, S. J., P. Eslin, et al. (2003). "Comparative study of the strategies evolved by two parasitoids of the genus Asobara to avoid the immune response of the host, Drosophila melanogaster." Dev Comp Immunol **27**(4): 273-282.

Moreira, S., B. Stramer, et al. (2010). "Prioritization of competing damage and developmental signals by migrating macrophages in the Drosophila embryo." Curr Biol **20**(5): 464-470.

Morel, O., N. Morel, et al. (2008). "Platelet microparticles and vascular cells interactions: a checkpoint between the haemostatic and thrombotic responses." Platelets **19**(1): 9-23.

Moreno, E., M. Yan, et al. (2002). "Evolution of TNF signaling mechanisms: JNK-dependent apoptosis triggered by Eiger, the Drosophila homolog of the TNF superfamily." Curr Biol **12**(14): 1263-1268.

Morrissey, J. H., V. Pureza, et al. (2008). "Blood clotting reactions on nanoscale phospholipid bilayers." Thromb Res **122 Suppl 1**: S23-26.

Mukae, N., H. Yokoyama, et al. (2002). "Activation of the innate immunity in Drosophila by endogenous chromosomal DNA that escaped apoptotic degradation." Genes Dev **16**(20): 2662-2671.

Myat, M. M. and D. J. Andrew (2000). "Organ shape in the Drosophila salivary gland is controlled by regulated, sequential internalization of the primordia." Development **127**(4): 679-691.

Nagata, S. (2007). "Autoimmune diseases caused by defects in clearing dead cells and nuclei expelled from erythroid precursors." Immunol Rev **220**: 237-250.

Nappi, A. J., Y. Carton, et al. (1991). "Parasite-induced enhancement of hemolymph tyrosinase activity in a selected immune reactive strain of Drosophila melanogaster." Arch Insect Biochem Physiol **18**(3): 159-168.

Nappi, A. J. and E. Vass (1993). "Melanogenesis and the generation of cytotoxic molecules during insect cellular immune reactions." Pigment Cell Res **6**(3): 117-126.

Nappi, A. J., E. Vass, et al. (1995). "Superoxide anion generation in Drosophila during melanotic encapsulation of parasites." Eur J Cell Biol **68**(4): 450-456.

Nappi, A. J., E. Vass, et al. (2000). "Nitric oxide involvement in Drosophila immunity." Nitric Oxide **4**(4): 423-430.

Nappi, A. J., E. Vass, et al. (2004). "The effects of parasite-derived immune-suppressive factors on the cellular innate immune and autoimmune responses of Drosophila melanogaster." J Parasitol **90**(5): 1139-1149.

Niethammer, P., C. Grabher, et al. (2009). "A tissue-scale gradient of hydrogen peroxide mediates rapid wound detection in zebrafish." Nature **459**(7249): 996-999.

Noth, I., M. E. Strek, et al. (2003). "Churg-Strauss syndrome." Lancet **361**(9357): 587-594.

Ostrand-Rosenberg, S. (2008). "Immune surveillance: a balance between protumor and antitumor immunity." Curr Opin Genet Dev **18**(1): 11-18.

Page-McCaw, A. (2008). "Remodeling the model organism: matrix metalloproteinase functions in invertebrates." Semin Cell Dev Biol **19**(1): 14-23.

Pages, F., J. Galon, et al. (2010). "Immune infiltration in human tumors: a prognostic factor that should not be ignored." Oncogene **29**(8): 1093-1102.

Pagliarini, R. A. and T. Xu (2003). "A genetic screen in Drosophila for metastatic behavior." Science **302**(5648): 1227-1231.

Pan, D. (2007). "Hippo signaling in organ size control." Genes Dev **21**(8): 886-897.

Pastor-Pareja, J. C., M. Wu, et al. (2008). "An innate immune response of blood cells to tumors and tissue damage in Drosophila." Dis Model Mech **1**(2-3): 144-154; discussion 153.

Pelte, N., A. S. Robertson, et al. (2006). "Immune challenge induces N-terminal cleavage of the Drosophila serpin Necrotic." Insect Biochem Mol Biol **36**(1): 37-46.

Philips, J. A., E. J. Rubin, et al. (2005). "Drosophila RNAi screen reveals CD36 family member required for mycobacterial infection." Science **309**(5738): 1251-1253.

Povelones, M., R. M. Waterhouse, et al. (2009). "Leucine-rich repeat protein complex activates mosquito complement in defense against Plasmodium parasites." Science **324**(5924): 258-261.

Prober, D. A. and B. A. Edgar (2000). "Ras1 promotes cellular growth in the Drosophila wing." Cell **100**(4): 435-446.

Ragab, A., T. Buechling, et al. (2011). "Drosophila Ras/MAPK signalling regulates innate immune responses in immune and intestinal stem cells." EMBO J.

Ramet, M., R. Lanot, et al. (2002). "JNK signaling pathway is required for efficient wound healing in Drosophila." Dev Biol **241**(1): 145-156.

Ramet, M., A. Pearson, et al. (2001). "Drosophila scavenger receptor CI is a pattern recognition receptor for bacteria." Immunity **15**(6): 1027-1038.

Read, R. D., W. K. Cavenee, et al. (2009). "A drosophila model for EGFR-Ras and PI3K-dependent human glioma." PLoS Genet **5**(2): e1000374.

Ribeiro, C. and M. Brehelin (2006). "Insect haemocytes: what type of cell is that?" J Insect Physiol **52**(5): 417-429.

Ricklin, D., G. Hajishengallis, et al. (2010). "Complement: a key system for immune surveillance and homeostasis." Nat Immunol **11**(9): 785-797.

Rizki, R. M. and T. M. Rizki (1980). "Hemocyte Responses to Implanted Tissues in Drosophila-Melanogaster Larvae." Wilhelm Rouxs Archives of Developmental Biology **189**(3): 207-213.

Rizki, R. M. and T. M. Rizki (1990). "Encapsulation of parasitoid eggs in phenol oxidase-deficient mutants of Drosophila melanogaster." Journal of Insect Physiology **36**(7): 523-529.

Rizki, T. M. and R. M. Rizki (1983). "Blood cell surface changes in Drosophila mutants with melanotic tumors." Science **220**(4592): 73-75.

Rizki, T. M., R. M. Rizki, et al. (1980). "A Mutant Affecting the Crystal Cells in Drosophila-Melanogaster." Wilhelm Rouxs Archives of Developmental Biology **188**(2): 91-99.

Robertson, A. S., D. Belorgey, et al. (2003). "Characterization of the necrotic protein that regulates the Toll-mediated immune response in Drosophila." J Biol Chem **278**(8): 6175-6180.

Russo, J., S. Dupas, et al. (1996). "Insect immunity: early events in the encapsulation process of parasitoid (Leptopilina boulardi) eggs in resistant and susceptible strains of Drosophila." Parasitology **112 ( Pt 1)**: 135-142.

Sackton, T. B., B. P. Lazzaro, et al. (2007). "Dynamic evolution of the innate immune system in Drosophila." Nat Genet **39**(12): 1461-1468.

Sanchez-Ferrer, A., J. N. Rodriguez-Lopez, et al. (1995). "Tyrosinase: a comprehensive review of its mechanism." Biochim Biophys Acta **1247**(1): 1-11.

Saraste, A. and K. Pulkki (2000). "Morphologic and biochemical hallmarks of apoptosis." Cardiovasc Res **45**(3): 528-537.

Sawamoto, K., P. Winge, et al. (1999). "The Drosophila Ral GTPase regulates developmental cell shape changes through the Jun NH(2)-terminal kinase pathway." J Cell Biol **146**(2): 361-372.

Scherfer, C., C. Karlsson, et al. (2004). "Isolation and characterization of hemolymph clotting factors in Drosophila melanogaster by a pullout method." Curr Biol **14**(7): 625-629.

Scherfer, C., H. Tang, et al. (2008). "Drosophila Serpin-28D regulates hemolymph phenoloxidase activity and adult pigmentation." Dev Biol **323**(2): 189-196.

Schlenke, T. A., J. Morales, et al. (2007). "Contrasting infection strategies in generalist and specialist wasp parasitoids of Drosophila melanogaster." PLoS Pathog **3**(10): 1486-1501.

Schmucker, D., J. C. Clemens, et al. (2000). "Drosophila Dscam is an axon guidance receptor exhibiting extraordinary molecular diversity." Cell **101**(6): 671-684.

Schneiderman, H. A. and E. Gateff (1967). "Control systems in insect development." Science **158**(3800): 534-535.

Secombe, J., S. B. Pierce, et al. (2004). "Myc: a weapon of mass destruction." Cell **117**(2): 153-156.

Senger, K., K. Harris, et al. (2006). "GATA factors participate in tissue-specific immune responses in Drosophila larvae." Proc Natl Acad Sci U S A **103**(43): 15957-15962.

Sentry, J. W., M. M. Yang, et al. (1993). "Conditional cell ablation in Drosophila." Bioessays **15**(7): 491-493.

Seong, S. Y. and P. Matzinger (2004). "Hydrophobicity: an ancient damage-associated molecular pattern that initiates innate immune responses." Nat Rev Immunol **4**(6): 469-478.

Shelby, K. S., O. A. Adeyeye, et al. (2000). "Parasitism-linked block of host plasma melanization." J Invertebr Pathol **75**(3): 218-225.

Sica, A., P. Larghi, et al. (2008). "Macrophage polarization in tumour progression." Semin Cancer Biol **18**(5): 349-355.

Sonnenfeld, M. J. and J. R. Jacobs (1995). "Macrophages and glia participate in the removal of apoptotic neurons from the Drosophila embryonic nervous system." J Comp Neurol **359**(4): 644-652.

Sorrentino, R. P. (2010). "Large standard deviations and logarithmic-normality: The truth about hemocyte counts in Drosophila." Fly (Austin) **4**(4).

Sorrentino, R. P., Y. Carton, et al. (2002). "Cellular immune response to parasite infection in the Drosophila lymph gland is developmentally regulated." Dev Biol **243**(1): 65-80.

Sorrentino, R. P., C. N. Small, et al. (2002). "Quantitative analysis of phenol oxidase activity in insect hemolymph." Biotechniques **32**(4): 815-816, 818, 820, 822-813.

Spector, W. G. (1976). "Immunologic components of granuloma formation. Epithelioid cells, giant cells, and sarcoidosis." Ann N Y Acad Sci **278**: 3-6.

Srivastava, A., J. C. Pastor-Pareja, et al. (2007). "Basement membrane remodeling is essential for Drosophila disc eversion and tumor invasion." Proc Natl Acad Sci U S A **104**(8): 2721-2726.

Staehling-Hampton, K., P. D. Jackson, et al. (1994). "Specificity of bone morphogenetic protein-related factors: cell fate and gene expression changes in Drosophila embryos induced by decapentaplegic but not 60A." Cell Growth Differ **5**(6): 585-593.

Stark, M. B. (1919). "A Benign Tumor that is Hereditary in Drosophila." Proc Natl Acad Sci U S A **5**(12): 573-580.

Steiner, H., D. Hultmark, et al. (1981). "Sequence and specificity of two antibacterial proteins involved in insect immunity." Nature **292**(5820): 246-248.

Steller, H. (2008). "Regulation of apoptosis in Drosophila." Cell Death Differ **15**(7): 1132-1138.

Stofanko, M., S. Y. Kwon, et al. (2010). "Lineage tracing of lamellocytes demonstrates Drosophila macrophage plasticity." PLoS One **5**(11): e14051.

Stramer, B., W. Wood, et al. (2005). "Live imaging of wound inflammation in Drosophila embryos reveals key roles for small GTPases during in vivo cell migration." J Cell Biol **168**(4): 567-573.

Stroschein-Stevenson, S. L., E. Foley, et al. (2006). "Identification of Drosophila gene products required for phagocytosis of Candida albicans." PLoS Biol **4**(1): e4.

Stuart, L. M. and R. A. Ezekowitz (2008). "Phagocytosis and comparative innate immunity: learning on the fly." Nat Rev Immunol **8**(2): 131-141.

Sullivan, W., M. Ashburner, et al. (2000). Drosophila protocols. Cold Spring Harbor, N.Y., Cold Spring Harbor Laboratory Press.

Tamori, Y., C. U. Bialucha, et al. (2010). "Involvement of Lgl and Mahjong/VprBP in cell competition." PLoS Biol **8**(7): e1000422.

Tang, H. (2009). "Regulation and function of the melanization reaction in Drosophila." Fly (Austin) **3**(1): 105-111.

Tang, H., Z. Kambris, et al. (2006). "Two proteases defining a melanization cascade in the immune system of Drosophila." J Biol Chem **281**(38): 28097-28104.

Tang, H., Z. Kambris, et al. (2008). "A serpin that regulates immune melanization in the respiratory system of Drosophila." Dev Cell **15**(4): 617-626.

Theopold, U., D. Li, et al. (2002). "The coagulation of insect hemolymph." Cell Mol Life Sci **59**(2): 363-372.

Theopold, U., O. Schmidt, et al. (2004). "Coagulation in arthropods: defence, wound closure and healing." Trends Immunol **25**(6): 289-294.

Tsan, M. F. and B. Gao (2004). "Heat shock protein and innate immunity." Cell Mol Immunol **1**(4): 274-279.

Uhlirova, M. and D. Bohmann (2006). "JNK- and Fos-regulated Mmp1 expression cooperates with Ras to induce invasive tumors in Drosophila." EMBO J **25**(22): 5294-5304.

Valanne, S., J. H. Wang, et al. (2011). "The Drosophila Toll signaling pathway." J Immunol **186**(2): 649-656.

Vidal, M. and R. L. Cagan (2006). "Drosophila models for cancer research." Curr Opin Genet Dev **16**(1): 10-16.

Vidal, S., R. S. Khush, et al. (2001). "Mutations in the Drosophila dTAK1 gene reveal a conserved function for MAPKKKs in the control of rel/NF-kappaB-dependent innate immune responses." Genes Dev **15**(15): 1900-1912.

von Landenberg, P. and S. Bauer (2007). "Nucleic acid recognizing Toll-like receptors and autoimmunity." Curr Opin Immunol **19**(6): 606-610.

Wajant, H., K. Pfizenmaier, et al. (2003). "Tumor necrosis factor signaling." Cell Death Differ **10**(1): 45-65.

Wang, Z., C. Wilhelmsson, et al. (2010). "Pathogen entrapment by transglutaminase--a conserved early innate immune mechanism." PLoS Pathog **6**(2): e1000763.

Waterhouse, R. M., E. V. Kriventseva, et al. (2007). "Evolutionary dynamics of immune-related genes and pathways in disease-vector mosquitoes." Science **316**(5832): 1738-1743.

Watson, F. L., R. Puttmann-Holgado, et al. (2005). "Extensive diversity of Ig-superfamily proteins in the immune system of insects." Science **309**(5742): 1874-1878.

Watson, K. L., T. K. Johnson, et al. (1991). "Lethal(1) aberrant immune response mutations leading to melanotic tumor formation in Drosophila melanogaster." Dev Genet **12**(3): 173-187.

Watson, K. L., R. W. Justice, et al. (1994). "Drosophila in cancer research: the first fifty tumor suppressor genes." J Cell Sci Suppl **18**: 19-33.

Wertheim, B., A. R. Kraaijeveld, et al. (2005). "Genome-wide gene expression in response to parasitoid attack in Drosophila." Genome Biol **6**(11): R94.

Wilder, E. L. and N. Perrimon (1995). "Dual functions of wingless in the Drosophila leg imaginal disc." Development **121**(2): 477-488.

Williams, M. J. (2007). "Drosophila hemopoiesis and cellular immunity." J Immunol **178**(8): 4711-4716.

Williams, M. J., I. Ando, et al. (2005). "Drosophila melanogaster Rac2 is necessary for a proper cellular immune response." Genes Cells **10**(8): 813-823.

Williams, M. J., M. L. Wiklund, et al. (2006). "Rac1 signalling in the Drosophila larval cellular immune response." J Cell Sci **119**(Pt 10): 2015-2024.

Wing, J. P., L. M. Schwartz, et al. (2001). "The RHG motifs of Drosophila Reaper and Grim are important for their distinct cell death-inducing abilities." Mech Dev **102**(1-2): 193-203.

Wing, J. P., L. Zhou, et al. (1998). "Distinct cell killing properties of the Drosophila reaper, head involution defective, and grim genes." Cell Death Differ **5**(11): 930-939.

Wood, W., C. Faria, et al. (2006). "Distinct mechanisms regulate hemocyte chemotaxis during development and wound healing in Drosophila melanogaster." J Cell Biol **173**(3): 405-416.

Wu, X., M. Yamada-Mabuchi, et al. (2008). "The Drosophila homolog of human tumor suppressor TSC-22 promotes cellular growth, proliferation, and survival." Proc Natl Acad Sci U S A **105**(14): 5414-5419.

Yamanaka, T. and S. Ohno (2008). "Role of Lgl/Dlg/Scribble in the regulation of epithelial junction, polarity and growth." Front Biosci **13**: 6693-6707.

Zeng, Q. and W. Hong (2008). "The emerging role of the hippo pathway in cell contact inhibition, organ size control, and cancer development in mammals." Cancer Cell **13**(3): 188-192.

Zettervall, C. J., I. Anderl, et al. (2004). "A directed screen for genes involved in Drosophila blood cell activation." Proc Natl Acad Sci U S A **101**(39): 14192-14197.

Zhang, L., T. Yue, et al. (2009). "Hippo signaling pathway and organ size control." Fly (Austin) **3**(1): 68-73.

Zhang, Q., M. Raoof, et al. (2010). "Circulating mitochondrial DAMPs cause inflammatory responses to injury." Nature **464**(7285): 104-107.

Zhao, B., L. Li, et al. (2010). "The Hippo-YAP pathway in organ size control and tumorigenesis: an updated version." Genes Dev **24**(9): 862-874.

Zhuang, Z. H., L. Sun, et al. (2006). "Drosophila TAB2 is required for the immune activation of JNK and NF-kappaB." Cell Signal **18**(7): 964-970.

Zwart, B., C. Ciurana, et al. (2004). "Complement activation by apoptotic cells occurs predominantly via IgM and is limited to late apoptotic (secondary necrotic) cells." Autoimmunity **37**(2): 95-102.

ISBN 978-3-86219-230-4