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THÈSE présentée par :

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Détection des protéases microbiennes par la voie immunitaire Toll chez *Drosophila melanogaster*

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II. Introduction à la thèse

La mouche du vinaigre, *Drosophila melanogaster*, est un petit insecte appartenant à l'ordre des Diptères, largement utilisé pendant le dernier siècle dans le but d'étudier différentes questions biologiques relatives, notamment à la neurobiologie et au développement embryonnaire. En raison de sa facilité d'élevage, de son cycle de reproduction relativement court (environ 10 jours à 25 °C), tout comme l'existence d'outils génétiques avancés, la drosophile est devenue un modèle d'étude de nombreux processus physiologiques, qu'elle permet d'analyser aux niveaux moléculaires et cellulaires.

Malgré les divergences évolutives (Kimbrell and Beutler, 2001), la drosophile partage avec l'Homme un nombre important de gènes homologues ainsi que des processus moléculaires aboutissant à l'activation de leurs systèmes immunitaires (Rubin and Lewis, 2000). Remarquablement, plus de 60% des gènes associés à des pathologies humaines ont des homologues chez la drosophile. Dépourvus de système immunitaire adaptatif conduisant à la production d'anticorps, les invertébrés combattent les agents infectieux uniquement par le biais de leur système immunitaire inné. Ainsi, la drosophile s'est avérée être un moyen pertinent permettant de décortiquer les mécanismes fondamentaux à la base de cette réponse immunitaire innée.

Dans la nature, la drosophile vit dans un environnement comportant des fruits en décomposition et riches en micro-organismes. Par conséquent, ces mouches sont constamment exposées à des microbes et sont menacées par différents pathogènes. Pour cette raison, elles ont dû développer durant l'évolution un système immunitaire robuste leur permettant de se défendre contre cette menace (Hoffmann and Reichhart, 2002). La première ligne de défense chez la drosophile se situe au niveau des sites d'entrée naturels des microbes: le tube digestif, les trachées et les organes génitaux (Lemaitre and Hoffmann, 2007). Quand les microorganismes entomopathogènes réussissent à traverser ces barrières épithéliales pour envahir la cavité interne, une deuxième ligne de défense se met en place au niveau de l'hémolymphe: elle consiste en la sécrétion principalement par le corps gras (l'équivalent fonctionnel du foie des

vertébrés) d'une batterie de peptides antimicrobiens (AMP), on parle alors de la réponse systémique humorale (Hoffmann, 2003).

Cette réponse systémique humorale peut être activée via deux voix de signalisation, les voies Toll et Immune Deficiency (IMD) (Ferrandon et al., 2007). Ces voies sont conservées pendant l'évolution et contrôlant l'expression et la sécrétion dans l'hémolymphe des AMP par les cellules du corps gras suite à une infection (Lemaitre and Hoffmann, 2007). La voie IMD est activée par des peptidoglycanes riches en acide diaminopimélique (DAP-type PGN) et communs aux bactéries à Gram-négatif (Leulier et al., 2003), et mène ainsi à l'expression d'un ensemble d'AMP comme la diptéricine et l'attacine, sous le contrôle du facteur de transcription de type NF-κB, Relish (Dushay et al., 1996). Quant au récepteur transmembranaire Toll, celui-ci n'interagit pas directement avec les déterminants microbiens, mais il est stimulé par l'interaction avec son ligand endogène, la cytokine Spaetzle (Spz), activée par une coupure issue d'un mécanisme protéolytique extracellulaire initié par une infection microbienne (Lemaitre et al., 1996). L'activation de la voie Toll aboutit à l'expression d'un ensemble différent d'AMP, dont la drosomycine, via un autre facteur de transcription de type NF-κB, Dorsal-related Immune Factor (DIF) (De Gregorio et al., 2002).

L'activation de Spz dépend de deux cascades protéolytiques distinctes. La première cascade est connue sous le nom de la voie des PAMP (Pathogen Associated Molecular Patterns). Comme son orthologue chez les mammifères, cette voie est en théorie basée sur la détection, par des récepteurs circulants, de composants spécifiques présents dans la paroi cellulaire des microorganismes fongiques et notamment les β-glucanes, ou des bactéries à Gram-positif et notamment le peptidoglycane de type lysine (Lys-type PGN) (El Chamy et al., 2008). Cette reconnaissance est suivie par l'auto-activation d'une protéase extracellulaire appelée ModSP (Modular Serine Protease) (Buchon et al., 2009), permettant ainsi l'activation séquentielle des Clip-protéases à sérine (Clip-SP) Grass et SPE (Spaetzle Processing Enzyme). Cette dernière est responsable de l'activation du ligand Spz (El Chamy et al., 2008; Kellenberger et al., 2011).

La deuxième cascade, connue sous le nom de « la voie des signaux de danger », dépend de la Clip-SP circulante Perséphone (Psh) et est activée suite à la perception d'activités protéolytiques anormales générées dans la cavité interne de la mouche par des bactéries à Gram-positif ou des champignons filamenteux (El Chamy et al., 2008; Gottar et al., 2006; Ligoxygakis et al., 2002).

Psh a été identifiée dans un crible ayant pour but d'identifier des suppresseurs du phénotype *necrotic*, qui est caractérisé par une activation constitutive de la voie Toll résultant de l'absence de Necrotic, un inhibiteur des protéases à serine (Levashina et al., 1999). Des études ultérieures ont montré que Psh est requise parallèlement à la voie des PAMP pour assurer une activation complète de la voie Toll en réponse à une infection par le champignon entomopathogène *Beauveria bassiana* ou par la bactérie à Gram-positif *Enterococcus faecalis*. En outre, une étude a démontré que la voie de signalisation dépendante de Psh est spécifiquement activée après l'injection des protéases purifiées à partir du champignon microscopique *Aspergillus oryzae* ou de la bactérie à Gram-positif *Bacillus subtilis*. Par ailleurs, cette voie est aussi induite suite à l'expression ectopique dans les mouches de la protéase Pr1A provenant du champignon *Metarhizium anisopliae*, et non par l'injection de PAMP purifiés. Etant donné que le zymogène de Psh (pro-Psh) est sensible à l'hydrolyse par la protéase Pr1A de *B. bassiana*, il a été proposé que Pro-Psh pourrait être le « détecteur » des activités protéolytiques anormales présentes dans l'hémolymphe des mouches suivant une infection (Gottar et al., 2006).

Les Clip-SP appartiennent à la même famille que celle de la chymotrypsine. Elles sont exprimées et secrétées sous forme de zymogènes inactifs et possèdent un pro-domaine N-terminal régulateur (domaine-Clip), connecté au domaine catalytique C-terminal par une séquence peptidique comportant 23 à 92 acides amines (Veillard et al., 2015). L'activation des Clip-SP dépend d'une coupure protéolytique à l'extrémité N-terminale du domaine catalytique, aboutissant à la libération de l'extrémité N-terminale d'une séquence consensus « IVGG- ». Suite à cette coupure, l'enzyme subit un changement de conformation permettant au domaine catalytique de devenir fonctionnel (Hedstrom, 2002; Kellenberger et al., 2011). Comme les Clip-SP sont organisées

en cascades, la spécificité d'une protéase devrait alors correspondre au site d'activation de la protéase qui la suit dans la cascade. Par conséquent, la majorité des Clip-SP chez la drosophile possèdent un résidu arginine ou lysine en position P1 de leur site d'activation (nomenclature de Schechter et Berger) (Schechter and Berger, 1967) afin qu'elles puissent être coupées par des protéases ayant une spécificité de type-trypsine (Ross et al., 2003). De manière notable, Psh se distingue de la majorité des Clip-SP en raison de la présence, en position P1, d'un résidu histidine qui est rarement accepté par le site de liaison au substrat des protéases à sérine. Ceci rend Psh théoriquement insensible à l'hydrolyse par la plupart des protéases.

Les bactéries et les champignons pathogènes produisent un large répertoire de protéases qu'ils utilisent comme facteurs de virulence. Certaines de ces protéases puissantes agissent d'une manière non-spécifique pour dégrader les protéines impliquées dans la défense immunitaire, tandis que d'autres sont intensément précises et agissent spécifiquement sur leur cible (Frees et al., 2013; Lebrun et al., 2009; Monod et al., 2002). Par conséquent, il serait difficile d'accommoder la haute précision requise pour l'activation de Psh à la diversité structurale et la large gamme de spécificité enzymatique des protéases microbiennes secrétées. Dans ce contexte, le but de ma thèse était d'examiner comment les signaux de danger peuvent manipuler le système immunitaire de l'hôte, et d'acquérir une meilleure connaissance du mécanisme moléculaire permettant la détection d'activités protéolytiques anormales par la protéase à sérine Psh.

J'ai commencé mon travail de thèse par un crible de différents pathogènes, bactéries et champignons, dans le but de vérifier leurs capacités à activer la voie Toll par l'intermédiaire de Psh. J'ai appliqué des conditions standardisées en utilisant des solutions microbiennes à concentrations bien définies, ainsi qu'un mode d'infection unique: la piqûre septique. Ensuite j'ai suivi l'activation de la voie Toll 16 heures après infection en mesurant le niveau d'expression de la *drosomycine*. En parallèle, je me suis servie d'un substrat non spécifique des protéases (AzoDye-collagène) afin de déterminer le niveau d'activité protéolytique extracellulaire libérée par chacun des micro-organismes

testés. Les résultats ont révélé la présence d'une corrélation rigoureuse entre le niveau d'activité protéolytique des différents pathogènes et leur capacité à activer la voie de danger. Ceci signifie que l'activation de la branche dépendante de Psh dans la voie Toll repose sur la présence de protéases microbiennes extracellulaires, indépendamment du type, de la structure ou de la spécificité des protéases secrétées.

J'ai également incubé une forme recombinante de pro-Psh, que nous avons purifiée à partir de cellules S2 de drosophile, avec différentes préparations microbiennes. Par *Western blot*, j'ai pu montré que l'hydrolyse de Psh se fait uniquement par des pathogènes possédant une activité protéolytique extracellulaire. Les résultats d'analyse par spectrométrie de masse révèlent que parmi tous les micro-organismes testés, seul *Bacillus subtilis* était capable de couper pro-Psh après le résidu histidine du site d'activation et de libérer l'extrémité N-terminale IVGG- nécessaire pour activer le domaine catalytique de Psh. D'une manière intéressante, la première coupure de Psh par tous les autres pathogènes a lieu dans une courte région du domaine-Clip (G_{107}-G_{115}) indiquant que cette séquence peptidique pourrait être considérée comme un « appât » peu spécifique pour la vaste majorité des protéases exogènes, et ce sans discrimination de leur origine, nature ou spécificité.

En effet, une coupure dans cette région ne suffit pas pour activer Psh, mais constitue la première étape d'une activation séquentielle. Ce mécanisme nécessite une protéase endogène capable de couper les résidus restants du domaine-Clip après l'histidine.

Afin de tester l'hypotèse de la présence d'une séquence « appât », nous avons construit des mutants de pro-Psh dans lesquels cette séquence était partiellement (rpro-Psh^{M1}) ou complètement (rpro-Psh^{M2}) substituée par des résidus alanine. *In vitro*, ces mutations ont réduit significativement la capacité de *B. subtilis* et de *E. faecalis* à couper à l'intérieur de cette séquence. Par ailleurs, nous avons exprimé ces deux mutants ainsi que la forme sauvage de pro-Psh dans des mouches mutantes nulles pour Psh sous la dépendance d'un promoteur spécifique du corps gras. L'expression de la forme sauvage a permis de restaurer

l'activation de la voie Toll chez les mutants Psh après l'infection des mouches avec *E. faecalis* ou bien l'injection de la protéase purifiée de *B. subtilis*. En revanche, l'expression des formes mutantes rpro-Psh^{M1} et rpro-Psh^{M2} dans les mouches mutantes pour Psh n'a pas permis de rétablir la capacité des mouches à exprimer la *drosomycine* suite à une infection par *E. faecalis*. Collectivement, ces résultats confirment le rôle crucial de la région « appât » dans l'activation de Psh par les protéases extracellulaires.

La deuxième partie de ma thèse a été consacrée à l'identification et la caractérisation de la protéase endogène potentiellement responsable de la deuxième étape d'activation de Psh. Parmi les protéases secrétées dans l'hémolymphe de la drosophile, les protéases à cystéine extracellulaires, et particulièrement les cathepsines, ont été décrites comme ayant des fonctions immunitaires chez les insects (Fujimoto et al., 1999). J'ai pu démontrer l'implication d'une protéase à cystéine dans la voie de Psh, et ceci en utilisant l'E-64, un composant possédant la capacité d'inhiber irréversiblement une large gamme de protéases à cystéine.

Afin d'identifier la protéase à cystéine endogène impliquée dans l'activation de la voie Toll en aval de Psh, j'ai effectué un crible ciblant les cathepsines exprimées normalement chez la mouche. J'ai pu mettre en évidence que l'insertion d'un élément-P dans le gène codant pour la cathepsine 26-29-p (*CG8947*) récapitulait le phénotype observé chez les mouches mutantes nulles pour Psh. Ensuite, en effectuant une excision imprécise de l'élément-P inséré dans l'exon 1 du gène *CG8947*, j'ai pu prouver que la cathepsine 26- 29-p est requise pour la deuxième étape d'activation séquentielle de Psh suite à une infection.

Pour conclure, nous avons pu montrer qu'à côté de la reconnaissance bien caractérisée de motifs moléculaires bien précis comme les PAMP par des récepteurs spécifiques (les récepteurs de patrons moléculaires ou PPR), les mouches ont développé un mécanisme leur permettant de détecter les activités enzymatiques générées par les micro-organismes envahisseurs. Ces enzymes microbiennes sont ensuite considérées comme « signaux de danger » activant la

voie Toll via la Clip-SP Psh. Tandis que l'activation de la voie des PRR repose sur une quantité élevée de pathogènes, la voie de reconnaissance des signaux de danger quant à elle permet une détection plus rapide et plus spécifique des micro-organismes secrétant des protéases.

Aussi nous avons identifié une région unique dans Perséphone qui fonctionne comme un appât pour toutes les protéases exogènes, et ce indépendamment de leurs origines. Une coupure dans cette région ne suffit pas à activer Perséphone, mais constitue la première étape d'une activation séquentielle: elle permet de recruter une autre molécule circulante de la famille des cathepsines (26-29p) (Issa et al., 2018). **Ces travaux montrent pour la première fois comment un récepteur de l'immunité innée, Perséphone, peut être activé par un signal de danger, en l'occurrence des protéases microbiennes**. Le mécanisme décrit permet une détection plus spécifique des infections que la détection de motifs moléculaires qui peuvent être présents dans la flore microbienne hébergée par les animaux.

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III. Table of Contents

IV. Abbreviations

Ala: Alanine

AMP: Anti-Microbial Peptide

AP: Alternative Pathway

APC: Antigen Presenting Cell

Asp: Aspartic acid

ATP: Adenosine Triphosphate

C1-INH: C1- inhibitor

C3aR: C3a ReceptorC4BP: C4b-binding protein

C5aR: C5a Receptor

C5L2: C5a receptor-like 2

CARS: Anti-inflammatory Response Syndrome

CCP: Complement Control Protein

Clip-SP: Clip-domain Serine Protease

CP: Classical Pathway

CR1: Complement Receptor 1

CR3, CR4: Complement Receptor 3,4

CRD: Carbohydrate-Recognition Domain

CRP: C-reactive protein

CrrY: Complement receptor 1-related gene/protein Y

Cys: Cysteine

DAF: Decay Accelerating Factor

DAMP: Damage-Associated Molecular Pattern

DAP-type PGN: meso-diaminopimelic acid-containing peptidoglycan

DC: Dendritic Cell

DD: Death-Domain

Deaf-1: Deformed epidermal autoregulatory factor-1

DIF: Dorsal-related Immune Factor

Dredd: Death-related ced-3/Nedd2-like protein

Drs: Drosomycin

Ea: Easter

Ecb: Extracellular complement-binding protein

EDTA: Ethylenediaminetetraacetic acid

Efb: Extracellular fibrinogen-binding protein

FADD: FAS Associated protein with Death-Domain

Gd: Gastrulation-defective

GNBP: Gram-Negative Bacteria Binding Protein

Gprk2: G-coupled receptor kinase 2

Grass: Gram-positive Specific Serine protease

HGF: Hepatocyte Growth Factor

His: Histidine

HP: Hematopoietic Pockets

Hsps: Heat shock proteins

I/R injury: ischemia-reperfusion injury

Ig: Immunoglobulin

IKK: Inhibitor of NF-κB (IκB) Kinase complex

IM1: Immune induced Molecule 1

IMD: Immune Deficiency

IRAK: IL-1 Receptor-Associated Kinase

IκB: Inhibitor of NF-κB

LDLa: Low-Density Lipoprotein receptor class A

LPS: Lipopolysaccharide

LRR: Leucine-Rich Repeat

Lz: Lozenge

MAC: Membrane Attack Complex

MASP: MBL-associated Serine Protease

MBL: Mannose-Binding Lectin pathway

MCP: Membrane Cofactor Protein

miRNA: micro RNA

ModSP: Modular Serine Protease

MP1, MP2: Melanization Protease 1 and 2

MPAC: truncated post-translationally modified pro-domain of attacin C

MyD88: Myeloid differentiation primary response 88

Ndl: Nudel

Nec: Necrotic

NF-κB: Nuclear Factor Kappa B

NLR: Nod-Like Receptor

PAMP: Pathogen-Associated Molecular Pattern

PAP: Prophenoloxidase-Activating Protein

PDV: Polyadnavirus

PGN: Peptidoglycan

PGRP: Peptidoglycan Recognition Protein

PO: Phenoloxidase

PPAE: Prophenoloxidase-Activating Enzyme

PPAF: Prophenoloxidase-Activating Factor

PPO: Prophenoloxidase

PRR: Pattern Recognition Receptor

Psh: Persephone

RCA: Regulators of Complement Activation

RCL: Reactive Central Loop

RHD: Rel-Homology Domain

ROS: Reactive Oxygen Species

RP49: Ribosomal Protein 49

Sbi: Surface immunoglobulin-binding protein

SCIN: Staphylococcal Complement Inhibitor

Ser: Serine

Serpin: Serine Protease Inhibitor

SIRS: Systemic Inflammatory Response Syndrome

sMAP: small MBL-Associated Protein

Smapins: small Serine Protease inhibitors

Snk: Snake

SP-like: Serine Protease-like

SP: Serine Protease

SpA: Surface-bound protein A

SPE: Spaetzle Processing Enzyme

SpeB: Streptococcal pyrogenic exotoxin B

SPH: Serine Protease Homolog

Spn: Serpin

Spz: Speatzle

SR: Scavenger Receptor

TAK1: Transforming Growth Factor beta (TGF-β)-Activated Kinase

TCC: Terminal Complement Complex

Thr: Threonine

TIR domain: Toll/IL-1 Receptor

TISC: Toll-Induced Signaling Complex

TLR: Toll-Like Receptor

TNF-α: Tumor Necrosis Factor-alpha

TNFR: Tumor-Necrosis Factor Receptor

ZPI: Protein Z-dependent inhibitor

α2-M: α2 macroglobulin

V. Innate immunity in *Drosophila melanogaster*

A. Foreword

The innate immune response of *Drosophila melanogaster* is governed by a complex set of signaling pathways that trigger phagocytosis, melanization, encapsulation and systemic antimicrobial peptides (AMPs) production through activation of the Toll and IMD pathways. The identification of the *Drosophila* Toll pathway cascade and the subsequent characterization of TLRs have reshaped our understanding of the immune system. Ever since, *Drosophila* NF-κB signaling has been actively studied. In flies, the Toll receptor is essential for embryonic development and immunity. In total, nine Toll receptors are encoded in the *Drosophila* genome, including the Toll pathway receptor Toll. The induction of the Toll pathway by invading microorganisms leads to the systemic production of specific AMPs. The Toll receptor is activated upon binding to its endogenous ligand Spaetzle (Spz), subsequently leading to the activation of the NF-κB factors Dorsal-related immunity factor (DIF) in adult flies or Dorsal in embryos and larvae. Here, I will describe the general features of the fruit fly innate immunity and I will focus on the current literature concerning the Toll pathway implication in the immune response of *Drosophila*.

B. General introduction: innate immunity

To protect themselves from diverse microbial threats, metazoans have established an innate immune response. This response protects the host from infection during the first critical hours of exposure to a new pathogen (Hoffmann, 2003).

In addition, vertebrates also established an adaptive immune response, which consists in the clonal expansion of lymphocytes after the specific recognition of a

given motif. This highly specific immune response, however, is not immediate and usually takes about four to seven days to get fully activated in humans (Charles A Janeway et al., 2001a, 2001b). By contrast, the innate immune response is immediate and of low specificity. It relies on a group of proteins and specialized cells that become quickly activated to help destroy invaders (Alberts et al., 2002).

In 1989, Charles A. Janeway Jr. proposed that innate immune detection relies on the presence of germline-encoded Pattern Recognition Receptors (PRRs) (Janeway, 1989). These receptors occupy both the extracellular and intracellular spaces and evolved to recognize conserved structural microbial components and determinants, such as peptidoglycans (PGNs) or lipopolysaccharides (LPS), produced by the invading pathogens, but not by the host (Medzhitov and Janeway, 2002). Recognition of these molecular structures, the so-called Pathogen-Associated Molecular Patterns (PAMPs), allows the immune system to distinguish infectious non-self from non-infectious self (Harris and Raucci, 2006; Janeway, 1992). Moreover, C. Janeway predicted that activation of the adaptive immune response is controlled by the more ancient innate immune system (Janeway, 1989).

In 1994 a new immunologic model suggested by Polly Matzinger stated that the immune system does not have the capacity to distinguish between self and non-self, but rather discriminates between safe and harmful. According to this theory, an immune response is not due to the presence of non-self but to the emission, within the organism, of « Danger Signals » by normal tissues. Upon stress or injury, damaged cells and tissues start to express and secrete Damage-Associated Molecular Patterns (DAMPs) that are recognized as « Alarm Signals » by PRRs (Matzinger, 1994, 2002). Danger signals are normal intracellular molecules among physiological conditions including DNA, RNA, heat shock proteins (Hsps), uric acid, Adenosine Triphosphate (ATP), and cytokines (interferon-α, interleukin-1β) (Brown and Lillicrap, 2002; Gallucci and Matzinger, 2001; Ishii et al., 2001).

The Danger model helped change our vision of adaptive and innate immunity. In the past, the innate immunity was suggested to be a minor part of the immune

system whereas the adaptive part was thought to be the most effective. As suggested by P. Matzinger's proposition, « the driving force behind immunity is the need to recognize danger » to allow the immune system to be alerted of danger and destruction without having to discriminate between self and non-self (Matzinger, 1994).

This new, competing theory has provoked mixed acclaim: it aroused much enthusiasm in scientific journals (Anderson and Matzinger, 2000; Gallucci and Matzinger, 2001; Gallucci et al., 1999; Matzinger, 2001, 2002, 2012), but also elicited reservations and criticism (Janeway Jr et al., 1996; Silverstein and Rose, 1997; Vance, 2000).

To assess the validity of the danger theory, it seems fundamental to define precisely what is a "danger," which appears problematic. Matzinger and her colleagues use several terms, that they interpret as equivalent: "danger," "damage," "stress," "injury," "necrosis," "inappropriate (non-physiological) cell death," etc. (Matzinger, 2002, 2007). Yet one can doubt that these terms are synonymous since, for instance, an organ transplant is not "dangerous" but rather useful for the receiver, yet Matzinger proposes that it is harmful because the surgeon's gesture damages the patient (Matzinger, 2002); in this case, it is not clear what counts as a "danger" for the organism. Furthermore, how does the notion of "danger" explain the immune responses to inoffensive antigens such as allergens or food antigens? In fact, the immune system inappropriately "sees" these as dangerous, even though they are not "really" dangerous. Thus, it is often unclear what a "danger" is for a cell or a tissue, and how cells and tissues can "perceive" that something is dangerous.

Matzinger' theory claims that every immune response is due to "damages" to the organism's cells or tissues. Indeed, it is easier to define what "damage" is (for an organism, a tissue or a cell) than what "danger" is (Anderson and Matzinger, 2000; Matzinger, 1994, 2002). Indeed, immune responses against "danger" are a theoretical suggestion, while the idea that they are due to "damages" has led to several experimental investigations. Therefore, in order to assess the "danger theory," the main concern is to define "damage" signals. Today, almost 20 years

after its first formulation, the danger theory is assessed towards recent experimental data on innate immunity, transplantation, cancers and tolerance to foreign entities (Pradeu and Cooper, 2012).

Recently, our lab showed that both, the PRR and Danger-based triggering of the immune reactions cooperate for a full activation of the innate immune response upon microbial challenges in *Drosophila* (El Chamy et al., 2008).

In mammals, the innate immune system, when abnormally regulated, contributes to a wide range of pathologies including chronic-inflammatory conditions, cancer and autoimmune diseases (Maeda and Omata, 2008). Chronic inflammation-related pathologies such as atherosclerosis, inflammatory-bowel diseases, polyarthritis or psoriasis are difficult to cure with currently available anti-inflammatory therapeutic molecules and are considered as a major health problem (Tabas and Glass, 2013). For this reason, deciphering the intricacies that govern the innate immune pathways is a considerable challenge for drug development, as understanding the fine-tuning events underlying the defense mechanisms cannot be dissociated from unraveling the next generation of therapeutic molecules.

Remarkably, the molecular mechanisms underlying the innate immune responses are highly conserved from insects to vertebrates (Janeway and Medzhitov, 2002; Medzhitov and Janeway, 2000).

C. General introduction: *Drosophila melanogaster*, an interesting model

Over the past century, the fruit fly *Drosophila melanogaster* has become a predominant model used to investigate complex biological issues, notably in neurobiology and developmental biology. The advanced genetic tools and the short generation time of *Drosophila* **(Figure 1)**, together with the fully sequenced genome (Adams et al., 2000) contributed to its success as a model organism. Importantly, *Drosophila* and humans share many genes and molecular pathways with similar functions (Rubin and Lewis, 2000). We know now that 75% of known human disease genes have a recognizable match in fruit flies. These diseases include Alzheimer's, Down's syndrom, autism, diabetes and cancers. "It's almost as if fruit flies were designed to help scientists," says geneticist Steve Jones in his book « Introducing Genetics: a graphic guide » (Jones, 2014). *Drosophila* is well suited for deciphering the fundamental mechanisms underlying the innate immune response as unlike in vertebrates its defense mechanisms rely entirely on innate immune responses. Although the final architecture of *Drosophila* and humans differs greatly, and they diverged more than 500 million years ago in evolution (Kimbrell and Beutler, 2001), many of the building blocks and the engineering processes underlying the activation of their innate immune systems are strikingly similar (Hoffmann and Reichhart, 2002). During the past three decades, massive efforts to describe the *Drosophila* innate immune system have largely contributed to the characterization of mammalian Toll-like Receptors (TLRs) and Nuclear Factor-Kappa B (NF-κB) (Leulier and Lemaitre, 2008) pathways, and demonstrated the relevance of this insect as a model to study innate immunity.

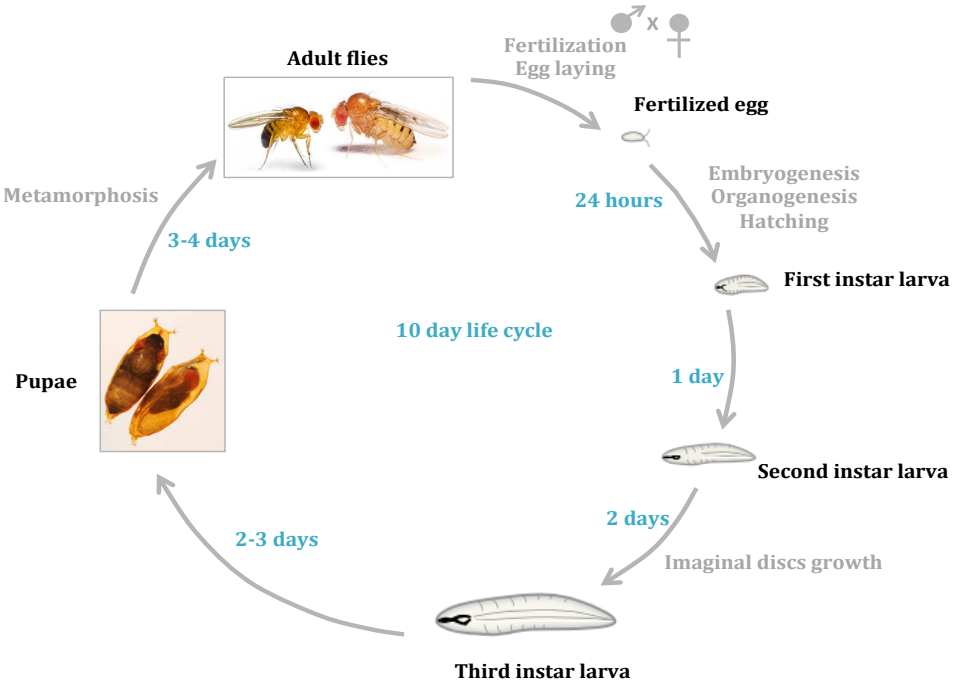


Figure 1: The life cycle of *Drosophila melanogaster*

Fruit flies are holometabolous insects: they undergo complete metamorphosis during their life cycle. The life cycle consists of four distinct stages: egg, larva, pupa, and adult. The rate of development depends on temperature, being more rapid at higher temperatures, which is a characteristic of all cold-blooded insects. When the flies are kept at room temperature (25 °C), the cycle lasts about 10 days.

Egg: A *Drosophila* female can lay up to 400-500 eggs in 10 days in a favorable laying ground (i.e., a decaying fruit). The egg is about 0.5 mm, ovoid, covered outside with a thin but strong chorion from which project anteriorly two thin stalks serving as "water-wings" to prevent the egg from sinking and drowning in a semi-liquid environment. At the anterior end of the egg is a minute pore (micropyle) through which the spermatozoa enter the egg as it passes down the oviduct into the uterus.

Larva: Within 24 hours of laying, the egg hatches into the 1st instar larva. The larva is a white, segmented, with black oral parts (jaw hooks) in the narrower head region. For tracheal breathing it has a pair of spiracles at both the anterior and posterior ends. Since insect cuticle will not stretch, the young small larva must periodically shed their cuticle in order to reach adult size. The larval stage of *Drosophila* consists of three instars. Within 24 hours of hatching, the larva molts to develop into 2nd instar larva. Again after 48 hours, this larva molts and matures to 3rd instar larva, which feeds until ready to pupate.

Pupa: After 3 days of feeding, the 3rd instar larva encapsulates itself inside a hard and pigmented puparium. It is in this pupal stage, where the metamorphosis of *D. melanogaster* takes place, giving rise to wings and legs. At room temperature conditions, the duration of metamorphosis lasts for 3-4 days.

Adult: adult flies emerge through the operculum of the puparium. They exhibit a typical insect anatomy, including compound eyes, a three-part body (head, thorax, and abdomen), wings, and six jointed legs. Within 8-12 hours of emergence, the female fly is receptive. Then, it mates with the male *Drosophila* for about 30 minutes, during which the male inseminates sperm inside the female fly. Mated females store sperm to fertilize eggs that are subsequently laid.

D. Immune responses in *Drosophila melanogaster*

In the wild, *Drosophila* lives in a microorganism-rich environment. As a consequence, flies are constantly exposed to microbial threats and had to develop a powerful immune system (Hoffmann and Reichhart, 2002).

1. The cellular immune response

The first arm of the *Drosophila* immune response consists in mesoderm-derived myeloid-like cells. *Drosophila* blood cells are produced by multipotent hematopoietic progenitors, called prohemocytes, that can give rise to three cell types collectively referred to as hemocytes, which participate in the elimination of the invaders through phagocytosis or encapsulation (Crozatier and Meister, 2007; Meister, 2004; Williams, 2007). Hemocytes comprise three cell types. Two among them are produced constitutively: the plasmatocytes, which are professional phagocytes and thus constitute the functional counterpart of mammalian macrophages, and the crystal cells, which are involved in microbial killing through the secretion of enzymes of the melanization cascade **(described in details in section V)** but also in blood coagulation as equivalent of mammalian platelets. The third cell type is lamellocytes, which appear only in parasitized larvae and are implicated in the encapsulation of a broad variety of intruders (El Chamy et al., 2017; Lanot et al., 2001; Vlisidou and Wood, 2015). *Drosophila* hematopoiesis occurs in two distinct waves (Holz et al., 2003). The first wave takes place early during embryogenesis when prohemocytes emerge from the procephalic mesoderm, migrate to colonize the embryo and differentiate mainly into plasmatocytes and into a few crystal cells under the control of the lineage-specific transcription factors Glide/Glial Cell Missing and the RUNX transcription factor Lozenge (Lz), respectively (Bataillé et al., 2005; Waltzer et al., 2010). Once specified, crystal cells, which represent around 5% of all embryonic blood cells, remain localized around the anterior part of the gut (proventriculus) (Lebestky et al., 2000), whereas plasmatocytes, which account for around 95% of blood cells, migrate under the influence of chemoattracting

signals to spread along embryonic tissues (Evans and Wood, 2014; Ratheesh et al., 2015; Wood et al., 2006). In the larval stages, these embryo-derived hemocytes then populate the larva in which they are found as circulating cells in the hemolymph (the circulatory fluid that fills the body cavity of Arthropods), or persist as patches of cells found along the dorsal vessel (a circulatory organ with heart-like function) and in the proventriculus, but also attached to specialized microenvironments of the subepidermal layers of the body cavity, the hematopoietic pockets (HPs) (sessile hemocytes) (Holz et al., 2003; Márkus et al., 2009; Petraki et al., 2015; Zaidman-Rémy et al., 2012).

A second wave of hematopoiesis occurs during the larval stages in a specialized organ, the lymph gland, which forms during embryogenesis (Crozatier et al., 2004). The lymph gland progenitors originate from an anlage of the thoracic mesoderm and proliferate until the second larval instar to form four to six bilaterally paired lobes along the anterior part of the dorsal vessel (Holz et al., 2003; Rugendorff et al., 1994).

The differentiation of lymph gland hemocytes is detected starting from the third larval stage. Under normal conditions, lymph gland prohemocytes give rise to crystal cells and plasmatocytes that do not leave the gland until the larva to pupa transition, when most of lymph gland cells differentiate before being released into the circulation as the lymph gland disrupts (Grigorian et al., 2011; Letourneau et al., 2016). In the case of parasitization of the larva by wasp eggs, the differentiation of prohemocytes is shifted to the production of lamellocytes (Honti et al., 2010; Jung et al., 2005). This reveals the considerable plasticity and concerted action of the hematopoietic compartments and the hemocyte lineages in the development of the innate immune system and in the course of the cell-mediated immune response in *Drosophila*.

2. The humoral immune response

The second arm of the *Drosophila* immune response is mainly executed by the fat

body, the functional equivalent of the mammalian liver. *Drosophila* humoral response mostly relies on the systemic secretion of Antimicrobial Peptides (AMPs) primarily by the fat body, and secondly on the local production of AMPs and Reactive Oxygen Species (ROS) at the level of epithelia (Ha et al., 2005).

a. Epithelial production of AMPs

The first layer of the *Drosophila* innate immune response is located at the putative entry sites of natural microbial infections: the gut, the tracheae and the genital plates (Lemaitre and Hoffmann, 2007). The barrier epithelia at these sites come into direct contact with microorganisms and thus need to prevent the penetration of pathogens that could cause systemic infections (Ferrandon et al., 1998; Önfelt Tingvall et al., 2001; Tzou et al., 2000). Recent findings in simple multicellular animals, such as *Hydra* and other *Cnidaria* (Bosch et al., 2009; Franzenburg et al., 2013; Mariottini and Grice, 2016), indicate that inducible expression of AMPs in epithelial cells constitutes an ancestral defense mechanism. AMPs are found in evolutionarily diverse organisms ranging from prokaryotes to invertebrates and vertebrates, and to plants (Bulet et al., 2004; Hancock, 2000; Tossi and Sandri, 2002). In *Drosophila*, the protective role of the barrier epithelia is twofold: it represents an impassable physical barrier, and provides a chemical barrier relying on the expression of immune-inducible AMPs and ROS (Davis and Engström, 2012).

b. Systemic production of AMPs

When entomopathogens cross the epithelial barriers and invade the internal cavity of the flies, a second layer of defense mechanisms is activated leading to the production and the release of over 20 AMPs belonging to eight gene classes into the hemolymph (Hoffmann, 2003; Rabel et al., 2004). These systemic responses were shown to be crucial for the defense against Gram-negative and

Gram-positive bacterial, viral and fungal infections (Braun et al., 1998; Kemp et al., 2013; Lemaitre et al., 1995a, 1996) and parasitoid wasp infestations (Carton and Boulétreau, 1985).

Upon infection, AMPs can reach a range of concentration from 0,5 μM (Diptericin) to 100 μM (Drosomycin) in the hemolymph, while their combined concentrations can reach 300 μM (Bulet et al., 1999; Meister et al., 2000), and may persist several weeks after the initial challenge (Imler and Bulet, 2005; Uttenweiler-Joseph et al., 1998). AMPs are generally between 12 and 50 amino-acid peptides including a large proportion of hydrophobic residues and at least two positively charged residues provided by arginine, lysine, or in acidic environments, histidine (Zhang and Gallo, 2016). These cationic peptides have a variety of antimicrobial activities extending from membrane permeabilization to action on a range of cytoplasmic targets (Mahlapuu et al., 2016). The initial contact between AMPs and their targets is electrostatic, as most bacterial surfaces are negatively charged or hydrophobic (Ebenhan et al., 2014; Gentilucci et al., 2006; Yeaman and Yount, 2003; Yeung et al., 2011). Their cationic charge, amino acid composition and size allow them to attach to membrane bilayers and to form pores (Bahar and Ren, 2013). Alternately, they may penetrate into the cell to bind crucial intracellular molecules (Hancock and Sahl, 2006). Individual AMPs selectively target different pathogens. Diptericin, Drosocin, Attacin and MPAC (truncated post-translationally modified pro-domain of attacin C) are active against Gram-negative bacteria. Defensin is important for clearing Gram-positive bacteria. Drosomycin and Metchnikowin are needed to fight fungal infections. Finally, Cecropins have both antibacterial and antifungal properties (Bulet et al., 1999). An additional AMP, Andropin, which is not immune-inducible, is expressed constitutively in male reproductive organs (Samakovlis et al., 1991).

Two evolutionary conserved NF-κB signaling pathways, Toll and immune deficiency (IMD), control the expression of the AMPs by fat body cells and their subsequent secretion in the general body cavity upon immune challenge (Ferrandon et al., 2007; Hoffmann, 2003).

i. The IMD pathway in *Drosophila melanogaster*

The *Drosophila* IMD pathway is often compared with the Tumor Necrosis Factor Receptor (TNFR) pathway (Myllymäki et al., 2014), one of the primary pathways involved in inflammation in mammals (Locksley et al., 2001) **(Figure 2)**. The IMD pathway is specifically triggered by the recognition of Gram-negative bacteria. To detect these microbes, the IMD pathway uses specific PRRs, the so-called Peptidoglycan Recognition Proteins (PGRPs) that bind to meso-diaminopimelic acid-containing peptidoglycan (DAP-type PGN) common to most Gram-negative bacteria (Leulier et al., 2003). The transmembrane receptor PGRP-LC and the secreted member of the PGRP family, PGRP-LE, are both required for sensing Gram-negative bacterial infections (Neyen et al., 2012). Similarly to Tumor Necrosis Factor-alpha (TNF-α), which exerts its effects through direct binding to its cognate receptor TNFR, PGRP-LC allows a direct recognition of Gram-negative bacteria at the surface of fat body cells. Upon binding to DAP-type PGN, PGRP-LC Death-Domain (DD) recruits a signaling complex consisting of the adaptor proteins Immune Deficiency (IMD) (a DD protein homolog of mammalian RIP1), drosophila FAS associated Death Domain (dFADD) (homolog of mammalian FADD) and the Caspase-8 homolog Death-related ced-3/Nedd2-like protein (Dredd). In both systems, Dredd and Caspase-8 can also promote apoptosis **(Figure 2)**. Upon activation, IMD and its homolog RIP1 recruit drosophila Transforming Growth Factor-beta (TGF-β)-Activated kinase 1 (dTAK1) and its homolog TAK1, respectively, which in turn activate the Inhibitor of NF-κB (IκB) Kinase (IKK) complex. In *Drosophila*, this IKK complex includes *Drosophila* homologs of IKKβ and IKKγ (NF-κB Essential Modulator, NEMO), Ird5 and Kenny respectively (Silverman et al., 2000). Activation of the IMD pathway and TNFR pathway target genes requires activation of the transcription factors Relish and NF-κB respectively. Like the mammalian Rel protein p105 and p100, Relish is characterized by a composite structure, containing an N-terminal Rel-homology domain (RHD) linked to a C-terminal

inhibitory ankyrin-reapeat domain characteristic of the IκB family (Dushay et al., 1996; Ferrandon et al., 2007; Hedengren et al., 1999).

Relish and NF-κB are activated by phosphorylation of the C-terminal inhibitory ankyrin-repeat region of Relish and the mammalian inhibitor IκB by the IKK complex. Subsequently, Relish and NF-κB are separated from their inhibitors by Dredd and Caspase-8 respectively, through and endoproteolytic cleavage. Stöven et al. demonstrated that the signal-dependent cleavage of Relish occurs at a caspase target site, and showed physical interaction between Relish and Dredd in transfected cells, confirming that Dredd is indeed the endoprotease needed for the activation of the pathway (Stöven et al., 2000).

The IκB-like C-terminal portion of Relish (Rel-49) and IκB remain in the cytoplasm while the N-terminal portions of Relish (Rel-68), which comprises the RHD, and NF-κB are imported to the nucleus (De Gregorio et al., 2002a), they bind to their cognate κB-response elements and activate the transcription of target genes, a specific set of AMPs (Dushay et al., 1996) and cytokines respectively.

Of note, the IMD pathway is highly regulated by a set of negative regulators acting at all levels of the pathway (Lee and Ferrandon, 2011). Removing one of these negative regulators, such as the amidase PGRP-SB, leads to a premature death of the flies (Paredes et al., 2011). This recapitulates the toxic effects observed during chronic inflammatory diseases in mammals, such as commensal dysbiosis (Guo et al., 2014).

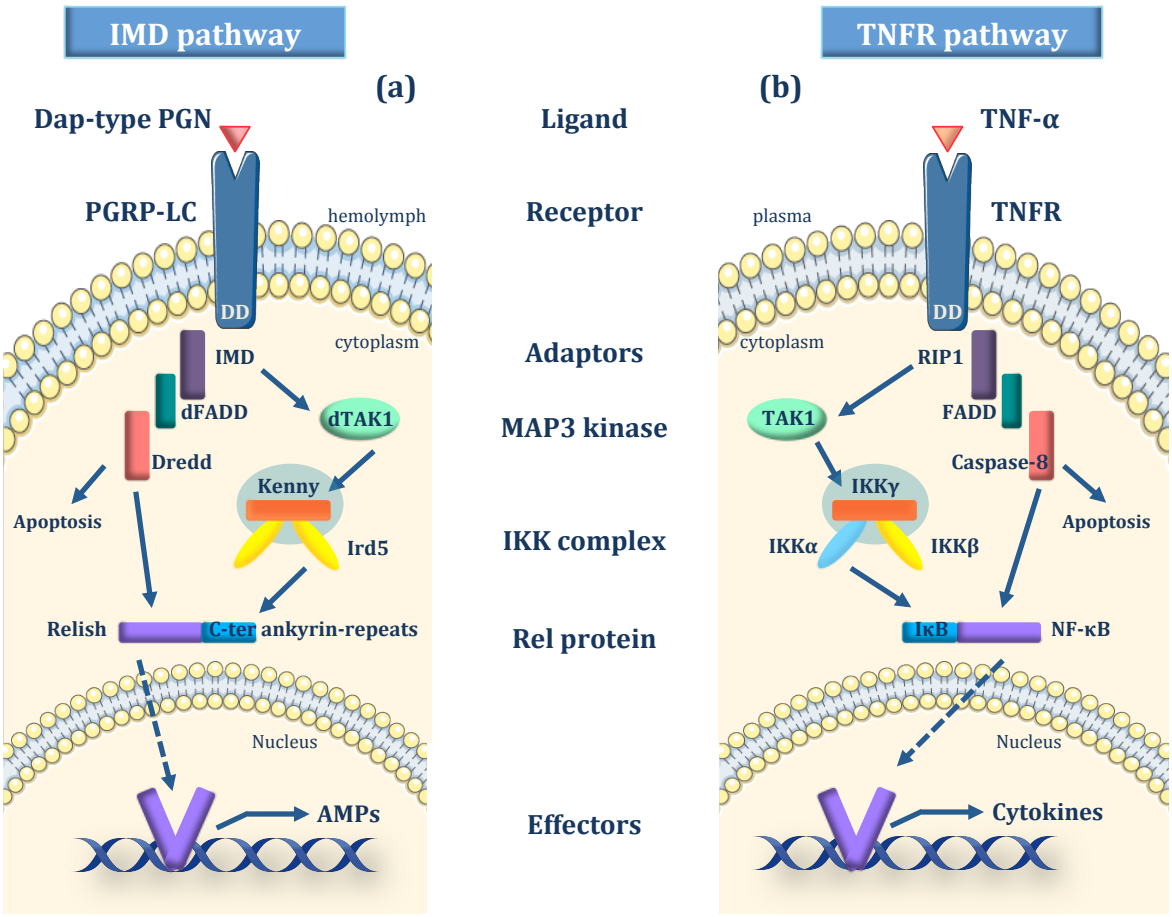


Figure 2: Schematic representation of the *Drosophila* IMD pathway and the mammalian TNF-α receptor (TNFR) signaling

(a) The IMD pathway in *Drosophila* governs defense reactions against Gram-negative bacterial infections. **(b)** Outlines of the mammalian TNF-α receptor (TNFR) signaling pathway, which highlights compelling similarities with the IMD pathway. Conserved components are indicated by similar shapes and colors.

The IMD pathway is specifically activated through the direct recognition of Gram-negative bacteria-derived diaminopimelic acid-containing peptidoglycan (DAP-type PGN) mainly by the Peptidoglycan-recognition protein PGRP-LC, similarly to the direct binding of TNF-α to its cognate receptor TNFR. PGRP-LC death-domain (DD) recruits a signaling complex consisting of the adaptors proteins Immune deficiency (IMD) (a DD protein homologous to mammalian RIP1), drosophila FAS associated Death domain (dFADD) (homologous to mammalian FADD) and the Caspase-8 homolog Death related ced-3/Nedd2-like protein (Dredd). In both systems, Dredd and Caspase-8 can also promote apoptosis. Upon activation, IMD and its homolog RIP1 recruit drosophila Transforming growth factor beta (TGF-β)-activated kinase 1 (dTAK1) and its homolog TAK1 respectively, which in turn activate the Inhibitor of NF-κB (IκB) Kinase (IKK)

complex. Activation of the IMD pathway and TNF-α target genes require activation of the transcription factors Relish and NF-κB respectively, by phosphorylation of the C-terminal inhibitory ankyrin-repeat region of Relish and the mammalian inhibitor protein IκB by the IKK complex. Subsequently, Relish and NF-κB are separated from their inhibitors by Dredd and Caspase-8 respectively through proteolytic cleavage. The IκB-like C-terminal portion (Rel-49) and IκB remain in the cytoplasm while the N-terminal portions of Relish (Rel-68) and NF-κB are then imported to the nucleus, they bind their cognate κB-response elements and activate the transcription of target genes, AMPs and cytokines respectively.

ii. The Toll pathway in *Drosophila* immune response

ii) 1. The Toll receptor in the immune defense of *Drosophila*

By contrast to the recognition events in the IMD pathway where PGRP-LC directly binds to DAP-type PGN, the transmembrane receptor Toll does not directly recognize microbial determinants but instead, it is activated by the binding of its endogenous ligand, the activated form of the cytokine Spaetzle (Spz) (Lemaitre et al., 1996) **(Figure 3)**.

The *Toll* gene was initially identified in 1985 in a series of genetic screens for genes involved in *Drosophila* embryonic development (Anderson et al., 1985). These screens were based on the saturation mutagenesis developed by Christiane Nüsslein-Volhard and Eric F. Wieschaus who found that the *Toll* gene encodes a new kind of type I transmembrane receptor that acts in establishing the dorso-ventral axis in early embryos (Nüsslein-Volhard and Wieschaus, 1980). Moreover, they identified 11 other genes that encode factors acting upstream and downstream of Toll to control embryonic segmentation (i.e., the dorsal group of genes, including *Toll*, *tube*, *pelle*, *cactus*, the NF-κB homolog *dorsal*, and seven genes upstream of Toll). This work constituted the basis for the discovery of dorso-ventral patterning genes, including most of the known

members of the Toll pathway (Belvin and Anderson, 1996). C. Nüsslein-Volhard and E. F. Wieschaus, together with Edward B. Lewis, earned the Nobel Prize in Physiology or Medicine in 1995 for their work on *Drosophila*.

In 1996, Jules A. Hoffmann and colleagues, using *Drosophila* mutant strains for the known components of the dorso-ventral Toll pathway, demonstrated that this pathway is also instrumental for the innate immune response against Gram-positive bacterial and fungal infections. Upon activation, the Toll receptor leads to the expression of a specific set of AMP coding genes (Lemaitre et al., 1996). The transcriptional activation of the genes encoding the AMPs requires transcriptional activators of the Rel family, to which NF-κB belongs (Lemaitre et al., 1995b). The work performed by J. Hoffmann provided new insights into the defense mechanisms that organisms, from the most primitive to humans, employ against infectious agents.

In 1997, Ruslan Metzhitov and Charles Janeway at Yale identified a Toll-like receptor (TLR) in mammals as a *Drosophila* Toll homolog capable of activating NF-κB and allowing the synthesis of cytokines and co-stimulatory molecules (Medzhitov et al., 1997). Separate studies led by Shizuo Akira, Bruce A. Beutler and others discovered that the TLRs act as the principal sensors of infection in mammals (Akira et al., 2006; Beutler, 2002, 2004, 2005, 2009; Takeuchi and Akira, 2001; Yoshimura et al., 1999). To date, 10 mammalian TLRs have been reported (Takeuchi and Akira, 2010). By demonstrating the marked conservation of innate defense mechanisms between insects and humans, the work initiated by Hoffmann earned him the Nobel Prize for Physiology or Medicine together with Bruce A. Beutler and Ralph M. Steinman in 2011.

It had already been recognized in the early 1990s that striking similarities exist between the control of the dorso-ventral patterning by the Rel transcription factor Dorsal in *Drosophila* embryos and the activation of the Rel protein NF-κB by the cytokine interleukin-1 in mammals (Belvin and Anderson, 1996; Hoffmann et al., 1999; Kopp and Medzhitov, 1999). In the absence of stimulation, Dorsal and NF-κB are held into the cytoplasm by homologous ankyrin-repeat proteins, Cactus and IκB respectively (Imler and Hoffmann, 2001). When the

extracellular ligands Spz and IL-1 activate their cognate transmembrane receptors and trigger the intracellular signaling cascades, Dorsal and NF-κB are respectively dissociated from their cytoplasmic inhibitors. Toll in *Drosophila* and the IL-1 receptor in mammals share a C-terminal intracytoplasmic homology domain, referred to as Toll/IL-1 receptor or "TIR" domain, that associates with adaptor proteins, drosophila Myeloid Differentiation primary-response gene 88 (dMyD88) and MyD88 respectively, allowing the activation of the homologous protein kinases Pelle in *Drosophila* and the mammalian IRAK (IL-1 Receptor-Associated Kinase) (Kopp and Medzhitov, 1999).

The N-terminal ectodomain of the Toll protein does not carry any similarity to the extracellular immunoglobulin-like ligand-binding portion of the IL-1 receptor. Instead, it bears leucine-rich repeats (LRRs) that are found in molecules as diverse as proteoglycans, adhesion molecules, enzymes, tyrosine kinase receptors and G-protein coupled receptors (Gay and Gangloff, 2007; Hashimoto et al., 1988). LRRs are shared by a number of other molecules in *Drosophila* such as 18-wheeler (Toll-2) (Eldon et al., 1994), Kekkon (Musacchio and Perrimon, 1996), Chaoptin (Reinke et al., 1988), Connectin adhesion molecules (Meadows et al., 1994; Nose et al., 1992) and the Slit secreted protein implicated in the establishment and the rearrangement of specific connections in the nervous system (Zinn and Sun, 1999), and in the vascular control of the hematopoietic microenvironments through Robo receptors (Morin-Poulard et al., 2016). However, LRRs have no documented function in the innate immune response.

ii) 2. Toll-related genes in *Drosophila*

Toll is a transmembrane receptor (Hashimoto et al., 1988). In addition to Toll, eight related genes all encoding transmembrane receptors with extracellular LRRs and intracytoplasmic TIR domain are present in the *Drosophila* genome

(Ooi et al., 2002; Tauszig et al., 2000). Excluding Toll-9, which contains an individual cysteine-rich motif upstream of the LRRs, a situation similar to mammalian TLRs, Toll and Toll-related receptors contain two or more characteristic cysteine-rich motifs surrounding the LRRs (Bilak et al., 2003; Imler and Hoffmann, 2001). Most *Drosophila* Toll receptors are strongly expressed during embryonic and pupal development and are seemingly implicated in developmental activities (Tauszig et al., 2000; Yagi et al., 2010). The immune functions of Toll receptors have been analyzed. To date there are only mild indications that Toll-5 (Tehao) (Imler et al., 2000; Luo et al., 2001) and Toll-9 (Bettencourt et al., 2004; Ooi et al., 2002) could be involved in the activation of the Toll pathway, whereas genetic evidence reveals a role for Toll-8 (Tollo) in the negative regulation of the IMD pathway (Akhouayri et al., 2011). Collectively, these data support Toll as the main receptor involved upstream of the Toll pathway.

ii) 3. Signaling events of the Toll pathway downstream of the Toll receptor

Binding of the cytokine Spz to the N-terminal ectodomain of Toll triggers conformational changes in the receptor that license signaling (Gangloff et al., 2008; Hu et al., 2004; Weber et al., 2003). Activation of the pathway in adult flies implicates the assembly of a protein complex around the intracytoplasmic TIR domain of Toll. As in the TLR pathway, Toll requires the function of the adaptor protein dMyD88 composed of a death domain (DD) and a TIR domain (Horng and Medzhitov, 2001; Sun et al., 2002; Tauszig-Delamasure et al., 2002). Two other DD-containing proteins able to interact with each other, Pelle (an IRAK homolog that belongs to the family of serine-threonine kinases) (Shelton and Wasserman, 1993) and Tube (Letsou et al., 1991), form the Toll-Induced Signaling Complex (TISC) with dMyD88 **(Figure 3)**. The assembly of this complex is mediated by the bivalent DD of Tube, and leads to the Tube-dependent oligomerization and subsequent stimulation of the Pelle kinase

activity. Tube is not required any further in the following signaling steps (Ferrandon et al., 2007; Sun et al., 2004).

The TISC allows phosphorylation and subsequent degradation, through polyubiquitination, of the ankyrin-repeat inhibitor protein Cactus (IκB homolog) (Daigneault et al., 2013). The NF-κB homolog DIF (Dorsal-Related immunity Factor), retained in the cytoplasm by binding to Cactus, is consecutively translocated into the nucleus, triggering the expression of specific effector genes and antimicrobial peptides, among which Defensin, Drosomycin, and Metchnikowin (De Gregorio et al., 2002a; Wu and Anderson, 1998) **(Figure 3)**.

ii) 4. Intracellular regulation of the Toll pathway

Toll and TLR signaling pathways culminate in activation of the NF-κB/Rel transcription family, by nuclear translocation of cytoplasmic complexes. This activation plays a central role in the innate immune response through its ability to induce transcription of proinflammatory genes in mammals including cytokines, chemokines, immune effector genes and adhesion molecules (Baldwin, 1996). NF-κB-dependent responses need to be tightly controlled to avoid unwanted activation leading to runaway inflammation (Li and Verma, 2002). The Toll pathway, like the mammalian NF-κB-dependent inflammatory mechanisms can be regulated, positively or negatively, by a variety of molecules acting at different levels, going from the extracellular domain to the intracytoplasmic or even the intranuclear compartment.

The G-coupled receptor kinase 2 (Gprk2) is a conserved regulator of the Toll pathway is, which was found to be crucial for the defense against Gram-positive bacterial and fungal infections. In fact, *Gprk2* RNAi flies were shown to be more susceptible to infection with the Gram-positive bacterium *Enterococcus faecalis*, and exhibited reduced expression of Toll pathway target genes. Gprk2 is a positive regulator of that associates with Cactus in a kinase domain-dependent

manner, but is not required for Cactus phosphorylation and subsequent degradation (Valanne et al., 2010).

Moreover, Deformed epidermal autoregulatory factor-1 (Deaf-1), which was first identified as a transcription factor, is required downstream of DIF/Dorsal and binds the *metchnikowin* and *drosomycin* promoters in response to both bacterial and fungal infections (Kuttenkeuler et al., 2010; Reed et al., 2008).

Furthermore, SUMOylation plays a role in Toll pathway regulation. Bhaskar et al. identified the *Drosophila* SUMO-activating enzyme dUbc9 in a two-hybrid screen for Dorsal-interacting proteins. Ubc9 conjugates the ubiquitin-like polypeptide SUMO to various protein substrates. Experiments probing the impact of dUbc9 on Dorsal-mediated transcription showed that conjugation of SUMO to lysine 382 of Dorsal allows it to overcome Cactus-dependent cytoplasmic sequestration, thus enhancing its nuclear translocation (Bhaskar et al., 2000). dUbc9 was later shown by Chiu et al. to negatively regulate the Toll signaling and repress the expression of *drosomycin*, which is constitutively induced in dUbc9 mutant flies in the absence of immune challenge (Chiu et al., 2005).

Another intracellular regulator of the Toll pathway is Pellino. Separate studies showed that Pellino exerts a dual role in the regulation of the Toll pathway. Pelle has a C-terminal kinase domain but does not directly phosphorylate Cactus. Instead, Pelle autophosphorylates, leading to its dissociation from the TISC (Shen and Manley, 1998). The Toll pathway can be positively regulated by Pellino, a highly conserved component of the pathway that interacts with Pelle and was shown by Haghayeghi et al. to be required for normal Drosomycin expression and resistance against Gram-positive bacteria (Haghayeghi et al., 2010). It was originally isolated in a two-hybrid screen performed by Grosshans and colleagues and shown to bind the phosphorylated and activated Pelle kinase, in order to mediate its interaction with Cactus (Grosshans et al., 1999). Structural homologs are present in the genomes of nematodes, mouse, and human. It has been reported that mammalian Pellinos (Pellino-1, -2, and -3) bind phosphorylated IRAK upon activation of the TLR and IL-1R pathways (Lin et al., 2008; Moynagh, 2009). Pellino-2, the closest homolog to *Drosophila* Pellino, was

shown to mediate lysine 63-linked polyubiquitination of IRAK (Schauvliege et al., 2006). While lysine 48-linked polyubiquitination results in recognition by proteasomes and degradation, lysine 63-linked polyubiquitination likely licenses protein-protein interaction leading to protein kinase activation and NF-κB stimulation (Skaug et al., 2009).

Later, a study led by Ji et al. uncovered that activation of Toll signaling leads to accumulation of Pellino at the plasma membrane, in physical interaction with MyD88. Thus, Pellino plays the role of a negative regulator by promoting MyD88 lysine-48-polyubiquitination and its subsequent degradation. This reveals a mechanism by which a feedback regulatory loop involving MyD88 and Pellino controls Toll-mediated signaling, thereby maintaining homeostasis of the host innate immune system (Ji et al., 2014).

Additionally, a study initiated in our laboratory (manuscript in preparation), together with data obtained from the laboratory of Prof. Ligoxygakis indicate that microRNAs (miRNAs) could be another layer of negative regulation in the Toll pathway (Atilano et al., 2017).

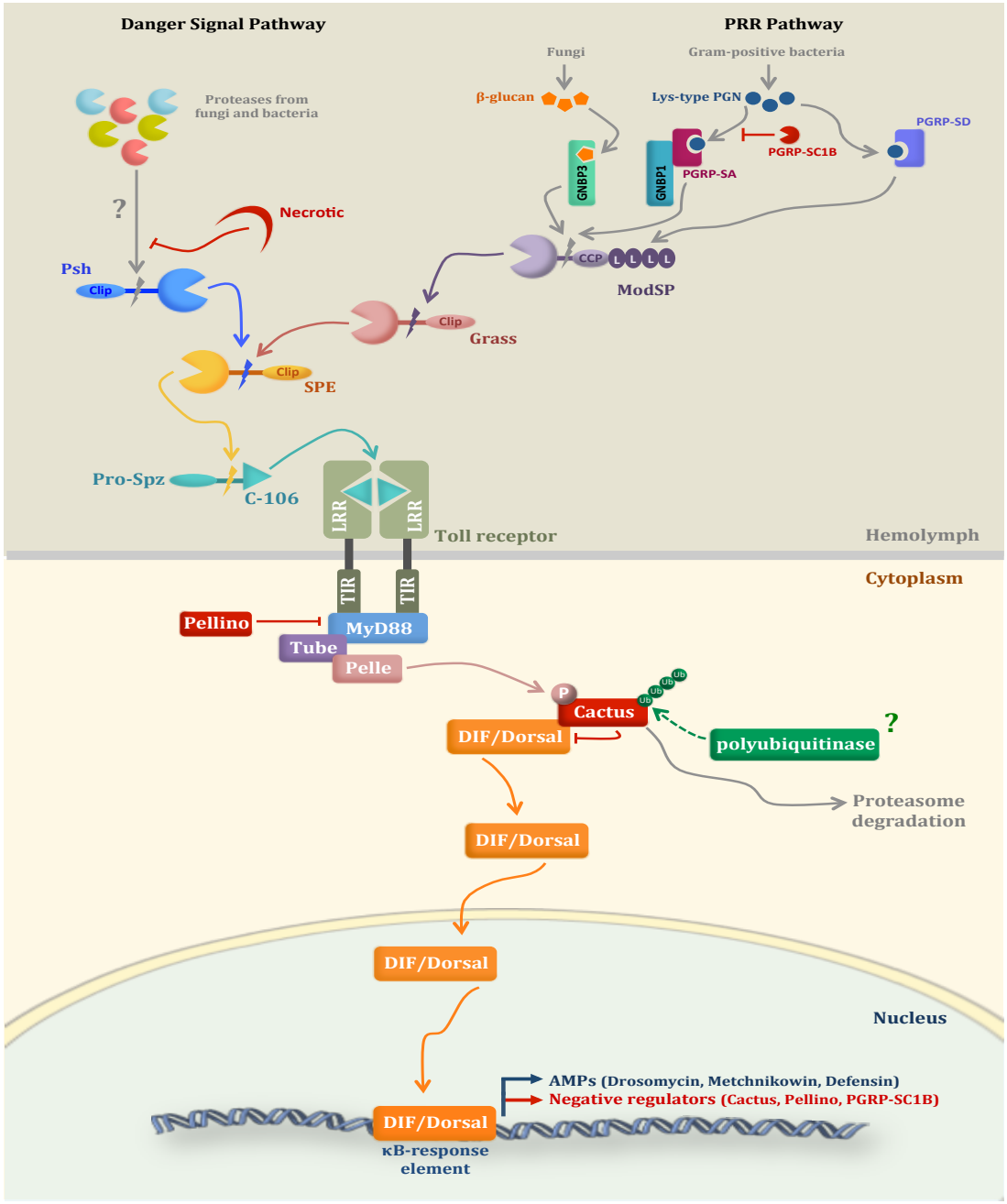


Figure 3: Schematic representation of the *Drosophila* immune Toll pathway

The Toll pathway is initiated by the recognition of Pattern-Associated Molecular Patterns (PAMPs) or danger signals through two parallel extracellular molecular cascades, Pattern Recognition Receptor (PRR) and Danger signals pathways respectively. In the PRR pathway, GNBP3 recognizes β-glucan from fungi while GNBP1/PGRP-SA complex recognizes Lysine-type PGN from Gram-positive bacteria. Alternatively, PGRP-SD also recognizes Lys-type PGN. Activated GNBP3, GNBP1/PGRP-SA complex and PGRP-SD further activate an extracellular proteolytic cascade involving the Modular Serine Protease (ModSP), and the Clip-domain Serine Proteases (Clip-SPs)

Gram-positive specific serine protease (Grass), and the Spaetzle processing enzyme (SPE), which cleaves and activates Spaetzle (Spz), the endogenous ligand of Toll. Alternatively, in the Danger signal pathway, SPE activation can be triggered by Persephone (Psh), another Clip-SP activated in the presence of proteases from virulent microorganisms, fungi, Gram-positive and Gram-negative bacteria. Activated SPE cleaves Spz, which undergoes a conformational change exposing its C-terminal or C-106 region. C-106 units bind as homodimers to the extracellular Leucin-Rich repeats of the Toll receptor. Once dimerized, the Toll/Interleukin-1 Receptor (TIR) intracytoplasmic domain of Toll recruits the adaptor proteins drosophila Myeloid differentiation primary response gene (88) (dMyd88), Tube and the Interleukin-1 receptor associated kinase 1 (IRAK1)-like Pelle kinase. Pelle further phosphorylates the IκB-like protein Cactus. Phosphorylated Cactus is then polyubiquitinated by Slimb and addressed to the proteasome for degradation. Degradation of Cactus releases the NF-κB factors Dorsal or Dorsal-related immunity factor (DIF) that translocate to the nucleus as homo or heterodimers. DIF/Dorsal dimers bind to their cognate κB-response element and activate the expression of Toll pathway target genes. DIF/Dorsal target genes include the antimicrobial peptides (AMPs) from the Drosomycin family, Metchnikowin and Defensin, and negative regulators retro-controlling the activation of the Toll pathway. Negative regulators of the Toll pathway are highlighted in red and are described in details in the main text.

ii) 5. Recognition events, extracellular mechanisms and regulation of the Toll pathway upstream of the Toll receptor

As mentioned above, activation of the Toll pathway requires binding of Toll receptor to its endogenous activated ligand Spz. In this section I will describe how recognition of Gram-positive bacteria or fungi results in the maturation of pro-Spz into a functional Toll receptor agonist **(Figure 3)**.

To activate the *Drosophila* Toll pathway in larvae or adult flies, extracellular recognition factors initiate proteolytic cascades in the hemolymph (Hoffmann and Reichhart, 2002). Cell wall components of both fungi (β-Glucan) and Gram-

positive bacteria (Lysine-type Peptidoglycan) are sensed by circulating receptors belonging to the family of PRRs (El Chamy et al., 2008). Recognition of Gram-positive bacteria is mediated by GNBP1, PGRP-SA and PGRP-SD, while GNBP3 is responsible for fungal detection (Gobert et al., 2003; Lee et al., 1996; Michel et al., 2001; Pili-Floury et al., 2004). Upon Gram-positive bacterial recognition, GNBP1 is activated and thus processes lysine-type PGN to new glycan reducing ends, which are presented to PGRP-SA. This leads to a physical interaction between GNBP1 and PGRP-SA followed by the formation of a complex between these two PRRs (Wang et al., 2006). In addition, PGRP-SD functions as a receptor for lysine-type PGN with partial redundancy to the GNBP1/PGRP-SA complex (Bischoff et al., 2004). Upon formation of this complex or upon fungal detection by GNBP3, a modular serine protease (ModSP), conserved in insects, is recruited to activate a downstream proteolytic cascade (Buchon et al., 2009). GNBP1 has been suggested to be a linker between PGRP-SA and ModSP (Wang et al., 2006). Stimulated ModSP will then activate the Clip-serine protease Gram-positive-specific serine protease (Grass) that in turn activates the Spaetzle Processing Enzyme (SPE) (El Chamy et al., 2008; Kellenberger et al., 2011). Grass was originally identified as acting specifically downstream of the recognition of the Gram-positive bacteria but was later shown to be also crucial for the detection of fungal components (El Chamy et al., 2008; Kambris et al., 2006).

Therefore, this PRR-mediated activation of the Toll immune pathway depends on the extracellular ModSP-Grass proteolytic cascade. It was reported by Fullaondo et al. that Serpin 1 (Spn1), belonging to the serpin superfamily, could negatively regulate this PRR-mediated dModSP-Grass proteolytic cascade at the level of GNBP-3. This study showed that expression of the Toll-dependent target genes *drosomycin* and *IM1* (Immune induced Molecule 1) is increased in *Spn1* null mutant flies. Moreover, the authors demonstrated that overexpression of Spn1 leads to decreased expression of *drosomycin* upon infection with the fungus *Beauveria bassiana* or the yeast *Candida albicans*, but not with the Gram-positive bacterium *Micrococcus luteus* (Fullaondo et al., 2011).

In addition to the PRR-mediated ModSP-Grass proteolytic cascade, a distinct extracellular proteolytic cascade leads to the activation of SPE independently of

the PRR complex GNBP1/PGRP-SA. This mechanism is mediated by Persephone (Psh), a Clip-serine protease, and is activated upon detection of danger signals such as fungal and bacterial virulence factors present in the hemolymph upon infection (El Chamy et al., 2008; Gottar et al., 2006). Of note, Ligoxygakis et al. originally isolated Psh in a screen for suppressors of the *necrotic* phenotype (discussed below), which is characterized by a constitutive activation of the Toll pathway resulting from the absence of the serine protease inhibitor Necrotic (Nec) (Ligoxygakis et al., 2002a).

Ligoxygakis and colleagues demonstrated that Psh is required for activation of the Toll pathway by the entomopathogenic fungus *Beauveria bassiana* (Ligoxygakis et al., 2002a). Importantly, it has been showed that Psh is needed in parallel to the PPR-mediated ModSP-Grass-SPE cascade for a full activation of the Toll pathway upon fungal infection or immune challenge with the Gram-positive bacterium *Enterococcus faecalis* (Gottar et al., 2006).

Several lines of evidence show that Psh could be a *bona fide* sensor for enzymatic activities, such as microbial secreted proteases. Gottar et al. proved that transgenic flies overexpressing the fungal *Metarhizium anisopliae* subtilisin *Pr1A* displayed a marked induction of Psh-dependent Toll pathway in the absence of infection. At the time, the mechanism allowing the detection of exogenous proteolytic activities was unclear. Thus, it has been proposed that the zymogen of Psh (pro-Psh) could act as a sensor for enzymatic activities (Gottar et al., 2006). However, the precision of the cleavage needed to activate Psh seems in contradiction with the high variability in specificity of the various proteases produced by pathogens (Veillard et al., 2015).

Along the same line, it has been reported by El Chamy et al. that Psh is required for activation of the Toll pathway, in absence of microbial motifs, by purified proteases from the filamentous fungus *Aspergillus oryzae,* or purified from the Gram-positive bacterium *Bacillus subtilis*. Collectively, this shows that Psh is a key sensor for abnormal enzymatic activities referred to as “Danger signals”. The group of Prof. Jean-Marc Reichhart also demonstrated that both, the Psh-based Danger Signal sensing and the PRR-mediated ModSP-Grass proteolytic cascade

converge to activate SPE upstream of the Toll receptor (El Chamy et al., 2008). This at last translates the Charles Janeway and Polly Matzinger's hypotheses into a single mechanism. Activated SPE in turn processes pro-Spz into a functional Spz. The cytokine Spz (Hoffmann and Reichhart, 2002) is able to form homodimers and presumably induces dimerization of Toll upon binding (Weber et al., 2003). In the absence of signaling, the N-terminal pro-domain of Spz masks the C-terminal or C-106 region (Arnot et al., 2010; DeLotto and DeLotto, 1998). Activation induces proteolysis of Spz by the Spaetzle Processing Enzyme (SPE), which causes conformational changes in Spz exposing determinants in the C-106 domain, critical for binding to the Toll receptor (Hu et al., 2004; Weber et al., 2003) **(Figure 3)**. The N-terminal pro-domain is released from C-106 upon Toll binding (Weber et al., 2007).

ii) 6. Extracellular regulation of the Toll pathway

At the extracellular level, during pattern recognition events, Lys-type PGN may be scavenged by the soluble PGRP-SC1B amidase activity (Mellroth et al. 2003) prior to PRR recognition, therefore preventing the activation of PRR recognition pathway.

Moreover, the Toll pathway is highly regulated by Serine Protease Inhibitors (Serpins) in order to prevent accidental activation of the serine protease cascade. The *Drosophila* genome encodes 29 serpins, most of unknown functions. In 1999, Levashina et al. showed that the circulating Necrotic (Nec) serpin, encoded by the *Spn43Ac* gene and synthesized in the fat body, negatively regulates the Toll signaling pathway. Loss-of-function *nec* mutants develop black spots along the body and the leg joints, corresponding to necrotic areas in the epidermis, and *nec* flies die within 3 or 4 days after eclosion (Levashina et al., 1999). Moreover, *nec* mutant flies exhibit a constitutive expression of *drosomycin* in the absence of immune challenge that is further enhanced upon infection. Later, Robertson et al.

reported that Nec has the specificity requirements to act as the physiological inhibitor of Psh *in vivo* (Robertson et al., 2003).

E. Conclusion

Despite the defense mechanisms that flies deploy at the epithelial barriers to prevent systemic dissemination of microbes, some succeed in invading the hemocoel. Consequently the adult flies trigger the systemic immune response. The cellular arm of the systemic response consists in circulating immune surveillance hemocytes (plasmatocytes, crystal cells and lamellocytes) that are active against microorganims and parasites (Crozatier and Meister, 2007). In addition to this cellular arm, induction of extracellular proteolytic cascades by circulating PRRs, or recognition of Gram-negative bacteria by PGRP-LC activate the humoral response that culminates in the secretion of AMPs secretion into the open circulatory system. It appears that since the discovery of Toll implication in the *Drosophila* development more than 30 years ago (Anderson et al., 1985), investigations at the molecular level have firmly settled the role of the Toll receptor in the immune response as well (Lemaitre et al., 1996). Several studies on recognition events and molecular mechanisms upstream of Toll, together with various screens targeting genes at the intracellular level, have uncovered many crucial components and conserved processes of the *Drosophila* Toll immune pathway. TLR and Toll pathways share many similarities; however, several differences exist (Imler and Hoffmann, 2001). For instance, while all TLRs belong to the family of Pattern Recognition Receptors, the Toll receptor is activated by a cytokine instead of microbial determinants (Gay and Gangloff, 2007). Moreover, all mammalian TLRs are involved in immune functions. In contrast, among the nine *Drosophila* Tolls, a clear immune function has only been assigned only to Toll itself.

The signaling transduction cascade leading, upon binding of Toll to its agonist Spz, to the nuclear translocation of the transcription factor NF-κB DIF is quite well understood. However, there are still open questions regarding the Toll

signaling pathway. First, it is still not known how DIF operates the transcription of its target genes, either alone or in combination with other factors similarly to what has been described for the NF-κB factor Relish (Bonnay et al., 2014; Goto et al., 2008). Secondly, only Cactus has been described as a potent negative regulator of the Toll pathway, in contrast to the IMD pathway that displays a variety of negative regulators. Therefore, it is likely that other negative regulators of the Toll pathway remain to be identified. Thirdly, at the beginning of my Ph.D. it was far from being clear how Psh could sense such a broad spectrum of microbial-based proteolytic activities, and how is Psh activated to subsequently trigger the danger arm of the Toll pathway. In fact, the capacity of Psh to be activated by a wide range of proteases seems a paradox, due to its biochemical properties, which makes it theoretically a quite poor target for other proteases. This was the starting point of my Ph.D. work.

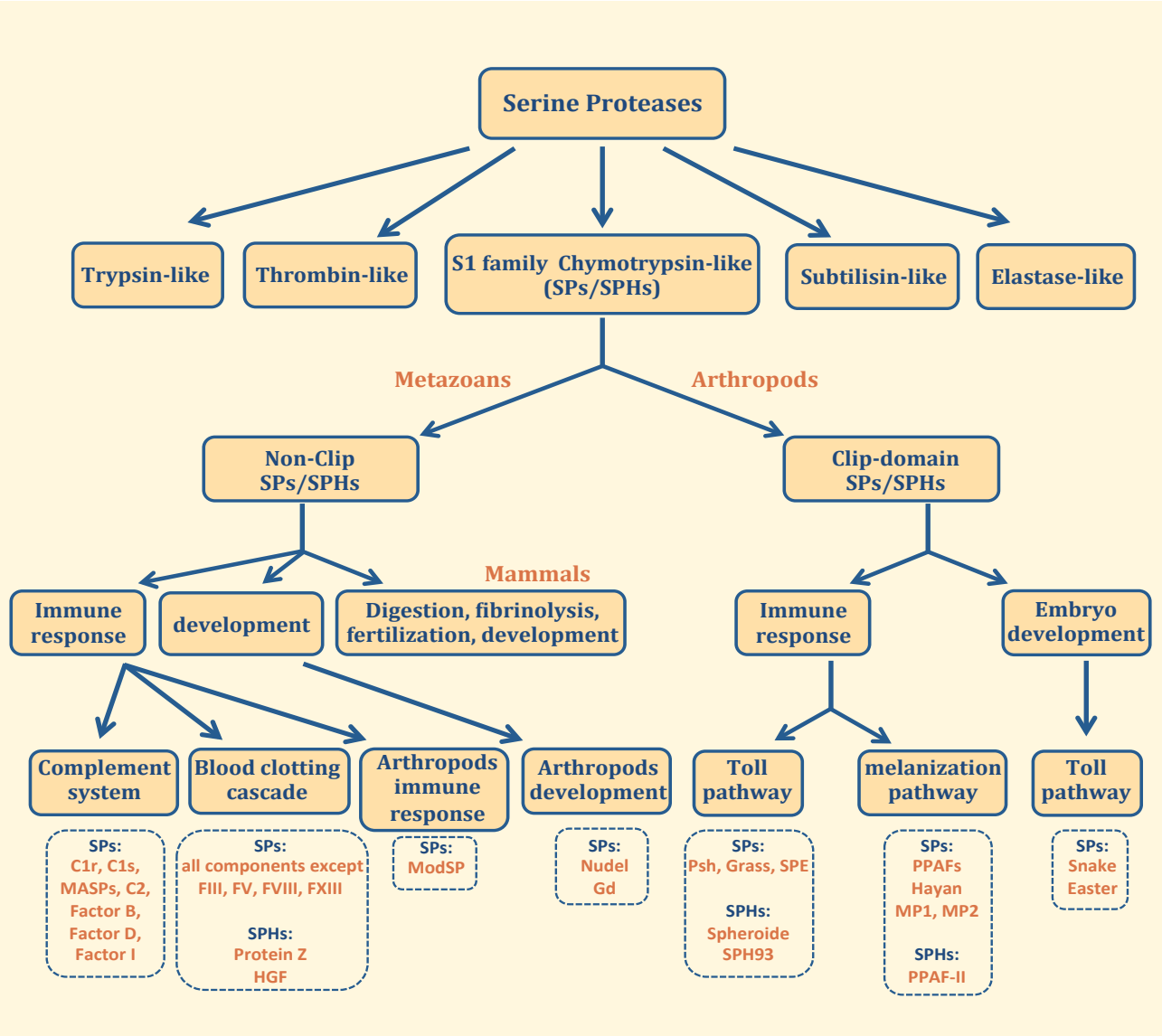


Figure 4: Classification of Clip *vs.* non-Clip Serine proteases (SPs) and Serine Protease Homologs (SPHs) in the animal kingdom

SPs and SPHs of the Chymotrypsin-like family, also known as the S1 family, are of the most thoroughly examined proteases. The active center of SPs consists of a catalytic triad comprising three amino acids: His-Asp-Ser. SPHs are related to SPs but are devoid of proteolytic activity because one or more residues of the catalytic triad are missing. Extracellular multi-domain SPs and SPHs are present in both Vertebrates and Invertebrates. The largest group of regulatory domains in Arthropods is the Clip-domain family.

SPs and SPHs of the S1 family are divided into two groups depending on the presence or the absence of a regulatory Clip-domain in the N-terminus of the protease. Conversely to non-Clip-SPs that are present in all metazoans, the Clip-domain is restricted to Arthropods and was not conserved during evolution.

In mammals, non-Clip SPs and SPHs are involved in a wide array of important physiological functions including digestion, fibrinolysis, fertilization, embryonic development and immune

responses. In insects, they have been reported to participate in development (Nudel and Gastrulation defective (Gd)) and in immune response (ModSP).

Clip-SPs and Clip-SPHs participate in three physiological processes in insects: the immne Toll pathway in response to microbial infections, the melanization pathway and the Toll pathway during embryonic development.

Properties and functions of SPs and SPHs are described in details in the main text below.

VI. Functions of Serine proteases (SPs) in the innate immune system

A. The S1 family: Chymotrypsin-like Serine Proteases (SPs) and Serine Protease Homologs (SPHs)

1. Chymotrypsin-like Serine Proteases (SPs)

Serine proteases (SPs) were among the first enzymes to be studied extensively. Chymotrypsin-like serine proteases, known as the S1 family, are one of the most thoroughly examined families of enzymes, which contributed highly to the development of structural and evolutionary biology, enzymology, as well as molecular physiology. This S1 family of SPs (Rawlings and Barrett, 1993) is implicated in a wide array of important physiological functions in mammals including digestion, blood coagulation, fibrinolysis, cellular and humoral immunity, fertilization and embryonic development. X-Ray crystal structure study of bovine chymotrypsin revealed that the active center of this enzyme consists of a catalytic triad, which comprises His57, Asp102 and Ser195 (Perona and Craik, 1995). Originally identified by Blow et al., these three residues of the central catalytic machinery are responsible for the acyl transfer mechanism of catalysis (Blow et al., 1969). Perona and Craik reported in 1997 that the catalytic Asp can adopt virtually any position with respect to the Ser-His dyad, suggesting that the classical « catalytic triad » of Ser, His and Asp residues can in fact be

better described as the juxtaposition of two dyads: Ser-His and His-Asp, that operate in concert (Perona and Craik, 1997). To prevent unwanted protein degradation, SPs are generally synthesized as inactive zymogens, which are converted to the active enzyme by proteolytic cleavage at a particular peptide bond (Khan and James, 1998; Neurath and Dixon, 1957). To achieve their functions, SPs are usually led by a secretion signal peptide to vesicular, granular, or extracellular locations. Zymogens of digestive SPs such as trypsin or chymotrypsin bear a short N-terminal peptide connected to the catalytic domain, whereas SPs playing a role in more complex events, such as development and immunity, are commonly multi-domain proteins (Ross et al., 2003). N-terminal pro-domains of extracellular SPs allow specific protein-protein interactions leading to formation of cascades in which one protease activates the subsequent zymogen to mediate a rapid, local physiological or pathological amplification of a small initial signal. After accomplishing their functions, the active enzymes are inactivated by serine protease inhibitors, especially members of the serpin superfamily **(described below in Section V)**.

The complement system activated in blood plasma upon microbial infection, as well as the blood-clotting cascade induced in response to tissue damage, are two classical examples of such extracellular SP cascades in mammals (Piao et al., 2005).

2. Serine Protease Homologs (SPHs)

Serine protease homologs (SPHs) are related to SPs and similar in amino acid sequence to S1 family, but seem to have no proteolytic activity because one or more of the catalytic residues are lacking (Ross et al., 2003). SPHs have been reported in vertebrates and invertebrates, although their exact functions are not very well understood. However, it is stated by Pils and Schultz that these catalytically dead molecules have acquired new regulatory roles that they may perform in a context dependent manner (Pils and Schultz, 2004). A first example

of mammalian SPHs that lack proteolytic activity is bovine Protein Z, a vitamin K-dependent plasma SPH, in which only Asp102 residue is present in the active site; His57 and Ser195 are replaced by Thr and Ala, respectively (Højrup et al., 1985). Bovine Protein Z was shown to interact with thrombin and phospholipid vesicles (Hogg and Stenflo, 1991). It has also been demonstrated that Protein Z binds with a high affinity to the Protein Z-dependent inhibitor (ZPI) to promote the inactivation of factor Xa of the coagulation cascade (Rezaie et al., 2008). A second example is human hepatocyte growth factor (HGF), which contains four kringle domains in the alpha subunit and shown to be highly homologous to SPs involved in blood coagulation and fibrinolysis, especially with plasminogen (Seki et al., 1991). HGF was suggested to play a role as a paracrine or endocrine mediator through an epithelial-mesenchymal interaction in wound healing, tissue regeneration, morphogenesis and carcinogenesis (Nakamura et al., 1989).

In the following sections, I will classify Both SPs and SPHs of the S1 family into two groups (Clip-SPs/SPHs; non-Clip-SPs/SPHs) depending on whether they carry a regulatory Clip-domain or not. This regulatory domain controls the proteinase action of various enzymes of the trypsin family. Conversely to non-Clip-SPs that are present in all metazoans, the Clip-domain is restricted to Arthropods and was not conserved during evolution **(Figure 4)**. It is always found in the N-terminal extremity of the SP and remains linked to the catalytic domain of the protease by a disulfide bridge after cleavage and activation. It is characterized by three conserved internal disulfide bonds. Clip-domain SPs and SPHs will be described in details later.

Among proteases, the non-Clip SPs emerged during evolution as the most abundant and functionally diverse group. They underwent predominant variations yielding the enzymes responsible for digestion, blood coagulation, fibrinolysis, development, fertilization, apoptosis, and immunity (Di Cera, 2009). Regarding their functions in the mammalian innate immune responses, non-Clip-SPs and SPHs have been reported to participate in the blood-clotting cascade and in the complement system. Non-Clip SPs have also been reported to play a role during development and innate immune repsonses in insects (**Figure 4)**.

In this next section, I will show one example of the SPs implication in the mammalian immune function by describing the main features of the mammalian complement system.

B. Example of Non-Clip Serine proteases in mammals: role in the complement system

1. The complement system activation pathways

The complement system in blood plasma is an integral part of the innate immune response in mammals, and acts as a bridge between innate and adaptive immunity (Carroll, 2000). Complement was first discovered in the 1890s by the bacteriologist George Nuttal as a heat-labile component of normal plasma, which recognizes, then augments the opsonization of bacteria by antibodies. This activity was said to aid or « complement » the antibacterial activity of antibodies, hence the name (Walport, 2001). Complement activation results in the generation of activated protein fragments that participate in microbial killing, phagocytosis, inflammatory reactions, antibody production and clearance of apoptotic cell debris and immune complexes from the circulation and damaged tissues as well (Davies et al., 1994; Mevorach et al., 1998; Schifferli et al., 1986). The complement system consists of a group of over 30 proteins and glycoproteins that are mostly synthesized in the liver and are either present as soluble proteins in the blood or as membrane-associated proteins (Morley and Walport, 2000).

Complement activation is known to occur through three different pathways: the classical, the alternative and the mannose-binding lectin pathways, each of them involves proteases, which are present as inactive zymogens that need to be sequentially activated. The non-Clip SPs of the complement system include C1r, C1s, MASPs (Mannose-binding lectin-Associated SPs), C2, Factor B, Factor D and Factor I (Sim and Laich, 2000) **(Figure 5)**.

Each complement activation pathway responds to a different set of activators, ensuring that a wide range of dangerous factors are recognized: the classical pathway is triggered by antibody-antigen complexes, the alternative pathway is activated by a battery of microbial surface components, and the mannose-binding lectin pathway responds to specific sugar moieties (Kemper and Hourcade, 2008). All three pathways converge at the complement component C3 (which is the most abundant complement protein found in the blood), resulting in the formation of the activation products C3a, C3b, C5a and then generate the membrane attack complex (MAC) C5b-9, leading to cytolysis (Sarma and Ward, 2011) **(Figure 5)**.

a. The Classical Pathway (CP)

The classical pathway (CP) is initiated when immune complexes are formed in the plasma after IgG or IgM binding to pathogens or to other nonself antigens. The C1 complex is a multimeric protein association consisting of C1q, C1r and C1s molecules. C1q, the recognition protein (Arlaud et al., 2002), binds to the Fc region of the IgG or IgM immune complex, leading to the activation of C1r, which in turn cleaves and activates C1s. Consecutively, the active serine protease C1s cleaves C2, which itself is a serine protease, and C4 allowing the assembly of the protease complex C4b2a (also called C3 convertase), which in turn cleaves C3 into C3a and C3b (Arumugam et al., 2006). While C3a acts as an anaphylatoxin that recruits inflammatory cells, C3b binds to complement-activating surfaces. Surface-bound C3b, and its break-down product iC3b, act as opsonins for phagocytes (Hurst et al., 1975) via the complement receptors CR1 (Ahearn and Fearon, 1989; Krych-Goldberg and Atkinson, 2001), CR3 and CR4, and help to further amplify complement activation. Besides, C3b binds covalently to the C4b portion of the C3 convertase C4b2a, to form the C5 convertase, C4b2a3b (Kim et al., 1992). The C5 convertase cleaves C5 to form C5a and C5b. The membrane attack complex (C5b-9, MAC), also called the terminal complement complex (TCC), is then initiated by C6 and C7 binding to C5b and then C8 and C9 binding to the C5bC6C7 complex. The MAC forms pores by inserting itself into the lipid bilayers of its targets, causing cell lysis (Morgan, 1999) **(Figure 5)**. The classical

pathway can also be activated by other danger signals like C-reactive protein (CRP) (Griselli et al., 1999; Padilla et al., 2007), viral proteins, polyanions, apoptotic cells and amyloid, thus providing evidence that the CP could be activated independently of antibodies (Barrington et al., 2001; Ehrnthaller et al., 2011; Gasque, 2004; Padilla et al., 2007).

b. The Alternative Pathway (AP)

For 50 years, complement was thought to function only in response to antigen-antibody complexes. That view began to change in 1954 thanks to work by Louis Pillemer and collaborators at the Western Reserve University (now Case Western University) (Pillemer et al., 1954). With the use of purified complement proteins, the alternative activation pathway was elucidated (Fearon, 1979; Pangburn and Müller-Eberhard, 1984).

The alternative pathway (AP) is triggered by carbohydrates, lipids and proteins expressed on foreign and nonself cell surfaces (Farries et al., 1988; Qu et al., 2009). The AP not only represents an individual recognition pathway, but also acts as an amplification loop of the classical and lectin pathways. As reported by Harboe and Mollnes, *in vivo* studies have elucidated that the AP alone can participate to >80% of the total activation mediated by either pathways (Harboe and Mollnes, 2008). In fact, there is a constant low-level induction of the AP following the spontaneous hydrolysis of C3 to C3(H_2O), which is called « tick-over ». This hydrolysis allows the formation of an initial AP C3 convertase C3(H_2O)Bb in the presence of the two serine proteases, Factor B and Factor D. Factor D cleaves then factor B to form the final C3 convertase C3bBb, which is stabilized by the presence of plasma properdin, also known as Factor P (Kemper et al., 2010). The C3 convertase from all pathways cleaves native C3 into two active fragments, the anaphylatoxin C3a and the opsonin C3b. The latter fragment binds covalently to nearby microbial surfaces. Properdin is a plasma component released by activated neutrophils. It is the only positive regulator of complement activation that stabilizes the convertase by binding to C3b (Hourcade, 2006) and preventing its cleavage by Factors H and I. Properdin was shown to act as a pattern-recognition molecule (Kemper and Hourcade, 2008) that binds to certain microbial surfaces, apoptotic cells and necrotic cells. Once

bound to a surface, properdin can direct convertase formation and initiate the AP activation (Spitzer et al., 2007).

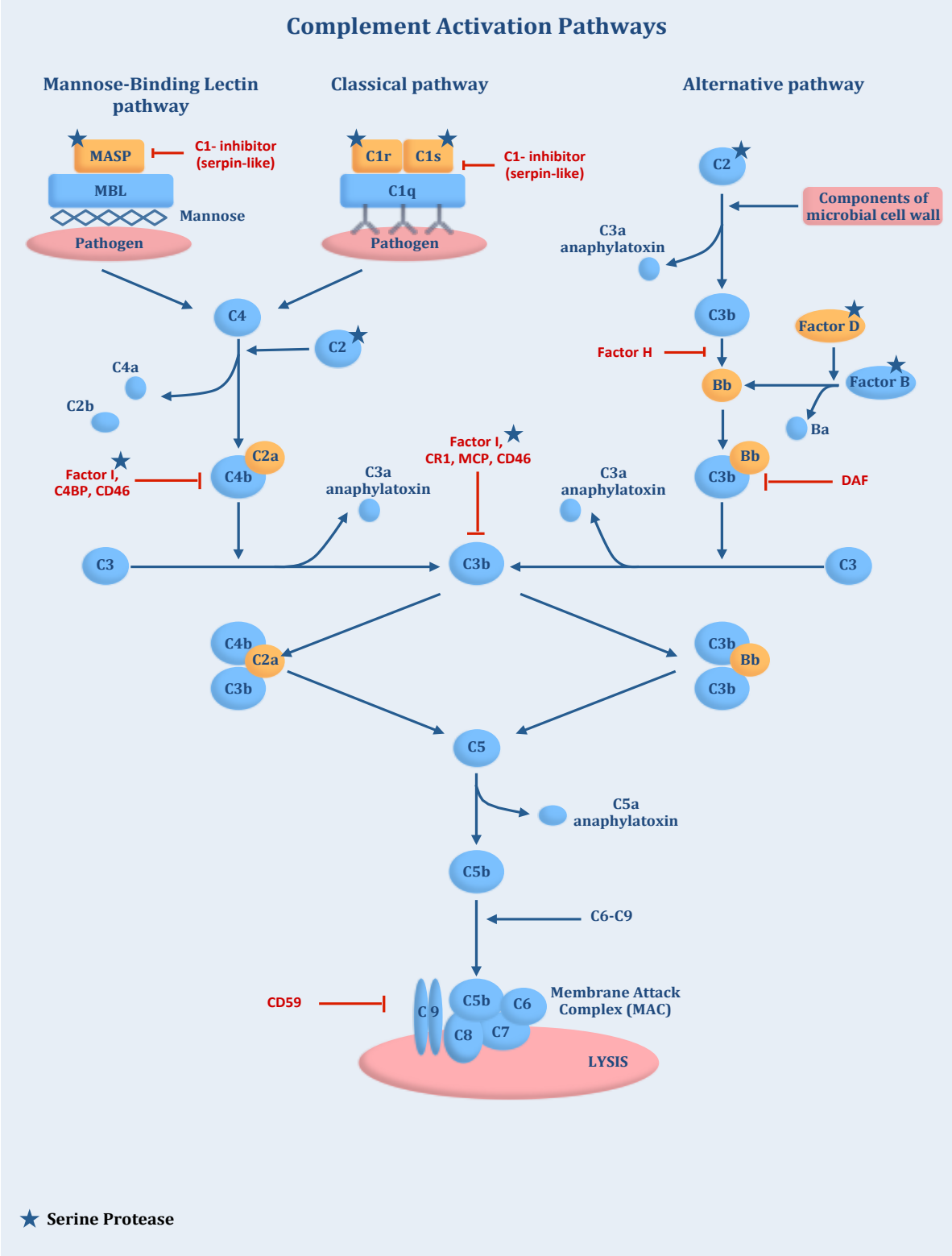


Figure 5: Schematic representation of the Complement activation pathways

(Described on the next page)

The complement system can be activated by three pathways: the antibody-dependent classical pathway, the alternative pathway, or by the mannose-binding lectin (MBL)/mannose-binding lectin-associated serine protease (MASP) pathway.

The classical pathway is initiated when IgM or IgG antigen-antibody complexes bind to the first component of complement system C1. The alternative pathway is triggered by C3b binding to various microbial activating surfaces. The MBL/MASP pathway is initiated by binding of lectin to specific carbohydrate patterns uncommon in the host. One characteristic of the alternative pathway is the slow generation of C3. The MBL/MASP pathway is initiated through the binding of MBL protein, associated with MASPs, to mannose residues on bacterial cell walls.

The three complement pathways consist in extracellular proteolytic cascades implicating serine proteases. The serine proteases of the complement system are C1r, C1s, MASPs, C2, Factor B, Factor D and Factor I, and are marked with a star in the figure. All pathways converge at C3 cleavage to initiate the formation of C3b, which mediates the formation of the C5 convertase. The C5 convertase initiates the assembly of the MAC, consisting of complement components C5b to C9. Activated MAC forms pores in the lipid bilayers of its targets, potentially causing cell lysis.

All three complement pathways allow the formation of anaphylatoxins C3a and C5a, which are robust mediators of inflammation.

The complement system is highly controlled by circulating and membrane-bound regulators that are highlighted in red in the figure. These negative regulators include: the soluble regulators Factor I, Factor H, C4b-Binding Protein (C4BP) or the serpin-like C1-inhibitor (C1-INH), and membrane-bound regulators such as CR1, CD46, DAF or CD59.

c. The Mannose-Binding Lectin pathway (MBL)

Forty years after the proposal of the alternative pathway, the MBL (mannose-binding lectin) pathway was discovered (Nesargikar et al., 2012). This complement pathway is initiated by the binding of MBL to carbohydrates of invading microorganisms. MBL contains a collagen-like domain and a carbohydrate-recognition domain (CRD). MBL circulates in the serum as

complexes with MBL-associated serine proteases (MASPs) in a Ca^{2+} dependent manner (Sørensen et al., 2005; Wallis, 2007). MASPs are involved in the cleavage of complement components (Matsushita and Fujita, 2001). Interestingly, MASP-proteases homologs, such as ModSP, have been found in invertebrates. This suggests that the MBL may be the first complement pathway to have arisen in evolution, considering that the CP and the AP have not been identified unambiguously in invertebrates (Sim and Laich, 2000). There are four structurally related MASPs (-1, -2 and -3) and a truncated MASP-2 known as MAP19 or small MBL-associated protein (sMAP) (Sørensen et al., 2005). Only MASP-2 has a clearly defined and sufficient role in triggering complement activation (Rossi et al., 2001). Binding of MBL-MASP-2 complex to pathogens induces conformational changes resulting in autoactivation of MASP-2, which in turn cleaves C4 and C2 to form C3 convertase **(Figure 5)**, while MASP-1 may cleave C3 directly bypassing the C4b2a complex, promoting the formation of C5 convertase, C4b2a3b (Presanis et al., 2003) albeit at a very slow rate (Hajela et al., 2002; Matsushita et al., 2000; Nesargikar et al., 2012). MASP-3 was shown to down-regulate the C4 and C2 cleaving activity of MASP-2 (Dahl et al., 2001). Three other lectin-type recognition proteins (PRRs), known as ficolins, have been demonstrated to interact with MASPs: ficolin-1 (or M-ficolin), ficolin-2 (or L-ficolin) and ficolin-3 (or H-ficolin or Hakata antigen). Ficolins activate the MBL pathway by forming active complexes with MASPs (Matsushita and Fujita, 2001; Matsushita et al., 2001). More recently, a new C-type lectin (collectin-11) was shown to bind to intact bacteria, fungi and viruses and to interact with MASP-1 and/or MASP-3. Hence, it is conceivable that collectin-11 plays a role in activation of the MBL pathway and in the defense against intruders (Hansen et al., 2010).

2. The complement system and inflammatory response

The anaphylatoxins C3a and C5a, which consist of 77 and 74 amino acids, respectively (Klos et al., 2009), exert a multitude of effects in pro-inflammatory responses. They are derived from the cleavage of the C parent components C3 and C5 respectively by the serine protease convertases during the activation cascade and they serve as potent chemoattractants for inflammatory cells such as phagocytes (neutrophils and monocytes) to sites of injury or inflammation (Sarma and Ward, 2011). Moreover, C3a and C5a are potent inflammatory mediators targeting a broad spectrum of immune and non-immune cells. They act as vasodilators, increase the permability of small blood vessels and induce the contraction of smooth muscles (Klos et al., 2009). C3a and C5a exert most of their pleiotropic effects by binding to their respective G-protein-coupled receptors, C3aR (Daffern et al., 1995; Fregonese et al., 2005; Gasque et al., 1998; Glovsky et al., 1979; Gutzmer et al., 2004; Ischenko et al., 1998; Klos et al., 1992; Monsinjon et al., 2003; Werfel et al., 2000; Zwirner et al., 1998) and in the case of C5a to two receptors, C5aR and C5a receptor-like 2 (C5L2) (Gao et al., 2005; Gavrilyuk et al., 2005; Laudes et al., 2002; Lee et al., 2001; Ohno et al., 2000; Okinaga et al., 2003; Riedemann et al., 2002; Wetsel, 1995).

It was demonstrated that C3a and C5a can be generated directly from C3 and C5, respectively by cysteine proteases found in the allergenic feces produced by dust mites (Maruo et al., 1997). Besides, *in vitro* findings suggested that the coagulation factors FXa, FXIa and plasmin could cleave both C5 and C3, leading to generation of anaphylatoxins C5a and C3a (Amara et al., 2008). Moreover, other serine proteases such as thrombin or plasma kallikrein are able to cleave and activate C3; similarly, C5 can be cleaved by thrombin, bypassing C3 (Ganter et al., 2007; Huber-Lang et al., 2002, 2006). This indicates possible cross-talks with other systems such as the coagulation cascade (Markiewski et al., 2007; Ricklin and Lambris, 2007).

3. Regulation of complement activation

Many distinct pathogenetic mechanisms may lead to the expression of an excessive and uncontrolled immune response. Depending on the individual's immune status, this could lead to non-infectious Systemic Inflammatory Response Syndrome (SIRS) or to Compensatory Anti-inflammatory Response Syndrome (CARS) (Ehrnthaller et al., 2011). In the case of uncontrolled complement activation, "friendly fire" is generated, resulting in the destruction of healthy host tissue including blood vessels (Acosta et al., 2004), kidneys (Thurman et al., 2005; Trouw et al., 2004), joints (Neumann et al., 2002) and erythrocytes (Holt et al., 2001). The activation of the CP and the MBL is largely dependent on foreign material, but in certain situations (eg, ischemia-reperfusion (I/R) injury), both pathways can be activated and cause autologous injury (Arumugam et al., 2004; Noris and Remuzzi, 2013). To protect against a complement attack, the human body has developed various strategies; both membrane-bound and fluid phase regulators participate to keep the complement system in check (Nesargikar et al., 2012) **(Figure 5)**.

Fluid phase or "soluble" regulators of complement activation (RCA) include Factor I (Fraczek and Martin, 2010), Factor H (Farries et al., 1990; Ferreira et al., 2010), C4b-Binding Protein (C4BP) (Fujita et al., 1985; Lappin and Whaley, 1990; Rawal et al., 2009), the anaphylatoxin inactivator Carboxypeptidase N (Bokisch and Müller-Eberhard, 1970; Matthews et al., 2004) and the C1-inhibitor (C1-INH). In the fluid phase, the best-known regulatory protein is C1-Inhibitor (C1-INH), which is synthesized in the liver and by monocytes. C1-INH forms an irreversible complex with the serine proteases C1r and C1s, typical of serpin regulation, and inactivates them. This leads to the disassociation of C1r and C1s from C1q in the complex. C1-INH can also bind to MASP-1 and MASP-2 and inactivate them leading to disruption of the MBL pathway (Fraczek and Martin, 2010; Vinci et al., 2002). Interestingly, this regulator acting at the level of SPs of the complement system uses a serpin-like inhibitory mechanism similar to the one described for *Drosophila* serpins that regulate SP cascades in development

and immune responses **(see below, section V)**.

Besides, the complement system is also regulated by membrane-bound RCAs that incude CD35 also know as Complement Receptor 1 (CR1) (Ahearn and Fearon, 1989; Miwa and Song, 2001), CD46 also known as Membrane Cofactor Protein (MCP) (Barilla-LaBarca et al., 2002; Cardone et al., 2011; Liszewski et al., 1991; Miwa et al., 1998), CD55 also known as Decay Accelerating Factor (DAF) (Lublin and Atkinson, 1989; Miwa and Song, 2001; Miwa et al., 2000; Song et al., 1996), CD59 (Davies et al., 1989; Meri et al., 1990; Rollins and Sims, 1990) and the Complement receptor 1-related gene/protein Y (CrrY) present only in rodents (Li et al., 1993).

4. Subversion of complement activity by pathogens

Many pathogenic organisms have evolved strategies to circumvent the capabilities of the complement system to eliminate them by dampening its activation. Although this carefully regulated cascade of enzymes, protein complexes and receptors ensures the rapid recognition and elimination of foreign structures, it also offers many sites of interference that can disrupt this balanced network of protein interactions. Pathogens accomplish this evasion by affecting every facet of the complement system events, including regulation of activation, amplification, opsonization, phagocytosis, chemoattraction and cell lysis (Lambris et al., 2008). Despite this plethora of complement-binding proteins, their mechanisms of action can be condensed to a few successful active strategies: **i) mimicking the host's regulatory proteins**, which is a strategy used by the Gram-negative bacterium *Borrelia burgdorferi*. *B. burgdorferi* is the causative agent of Lyme disease, which is one of the major emerging arthropod-borne pathologies in the world (Hengge et al., 2003). *B. burgdorferi* expresses on its membrane the "CD59-like" protein that binds in complex to C8b and C9 and inhibits MAC formation (Pausa et al., 2003). Notably, CD59-like protein binds preferentially to the C8β subunit, whereas human CD59 targets C8α.

ii) Recruitment of the host's RCAs (Blom et al., 2009; Zipfel et al., 2007), which is by far the most widely disseminated strategy to avoid the complement response. RCA recruitment is common in bacteria (for example, *Escherichia coli*, *Borrelia burgdorferi*, *Staphylococcus aureus* and *Streptococci*) (Blom et al., 2009; Ermert and Blom, 2016; Kraiczy and Würzner, 2006), but has also been described for fungi (for example, *Candida albicans*) (Meri et al., 2002, 2004) and parasites (for example, *Echinococcus*) (Inal, 2004).

iii) Microbial complement inhibition (Laarman et al., 2010; Lambris et al., 2008; Okumura and Nizet, 2014). A well known example of this strategy is the production of evasion molecules that target different levels of the complement system by the Gram-positive bacterium *Staphylococcus aureus*, which is a leading cause of nosocomial and community-acquired infections (Lowy, 1998). These molecules include the Extracellular fibrinogen-binding protein (Efb) and the Extracellular complement-binding protein (Ecb) (Jongerius et al., 2007, 2012), the Staphylococcal Superantigen-like 7 (SSL7) (Langley et al., 2005), the Staphylococcal complement inhibitor (SCIN) (Rooijakkers et al., 2005, 2009), the Surface immunoglobulin-binding protein (Sbi) (Haupt et al., 2008) and the surface-bound protein A (SpA) (Cedergren et al., 1993; Gouda et al., 1992).

iv) Cleavage of the complement components by the pathogens proteases, which is a widely used mechanism among bacteria (Potempa and Potempa, 2012). Examples of this strategy comprise the cleavage of C5a by the 56-kDa protease from the Gram-negative bacterium *Serratia marcescens* (Oda et al., 1990) or the C5a peptidases from *streptococci* (Chmouryguina et al., 1996). This cleavage efficiently disables C5a-mediated pro-inflammatory and chemotactic signaling leading to a disrupted immune response.

Streptococcus pyogenes is a Gram-positive bacterium responsible for a wide array of disease ranging from strep throat to life-threatening necrotizing fasciitis (Brouwer et al., 2016). It expresses streptococcal pyrogenic exotoxin B (SpeB), an endoprotease that can degrade several proteins including C3, immunoglobulin (Ig) and properdin, the positive regulator of complement activation. Upon cleavage, properdin loses its ability to stabilize the convertases on pathogenic surfaces (Honda-Ogawa et al., 2013; von Pawel-Rammingen and

Björck, 2003; Tsao et al., 2006).

A rare example of non-bacterial proteases that degrade components of the complement system has been observed in the parasitic worm *Schistosoma mansoni*, which degrades iC3b using a 28-kDa protease, resulting in impaired binding to CR3 (Ghendler et al., 1994).

In addition to these active strategies, many microorganisms also established passive evasive features; a prominent example is the thick peptidoglycan layer of Gram-positive bacteria cell wall, which prevents lysis by the MAC (C5b-9 complex) (Joiner et al., 1983).

The cleavage of the complement system components by virulence factors of *Serratia marcescens*, *Streptococcus pyogenes*, and other bacteria (not mentioned here) is an example of how microbes in general and particularly bacteria make use of their secreted proteases in order to degrade the host's proteins and inhibit the activation of immune reactions.

C. Serine Proteases (SPs) and Serine Protease Homologs (SPHs) in Arthropods

1. General Statements

Similarly to mammals, Arthropods also possess extracellular multi-domain SPs. In each insect species with a known genome, Clip and non-Clip SPs and SPHs constitute a large protein family with 50 to 300 members. Cao et al. reported that in *Manduca sexta* many of these SPs carry cystine-stabilized structures essential for molecular recognition and protein-protein interactions and which are already identified in mammals, including the low-density lipoprotein receptor class A repeats (LDLa), the scavenger receptor (SR), the Sushi, the Wonton, or the Frizzle domains, etc… (Cao et al., 2015). The largest group of regulatory modules in Arthropod SPs is the Clip-domain family. The prototype of the Clip-domain was initially identified in proclotting enzyme from the horseshoe crab *Tachypleus tridentatus* (Muta et al., 1990), and Clip-domains were so named by Iwanaga's group because a diagram of the disulfide linkages pattern in the N-terminal domain of this enzyme resembled a paper clip (Iwanaga et al., 1998). Biochemical and molecular biological analysis indicated that proclotting enzyme and other members of this protein family embrace two parts, a regulatory amino-terminal Clip-domain and a catalytic serine proteinase domain at the carboxyl terminus. A linking sequence, which varies in length between 23 and 92 amino acid residues, connects the two domains. An additional pair of cysteine residues links the two domains such that when the protease is activated, the catalytic heavy chain remains covalently attached by a disulfide bond to the light chain, which contains the Clip-domain (Jiang and Kanost, 2000).

Clip-domain serine proteases (Clip-SPs) have been identified in other arthropods, where they participate in embryonic development and immune responses, including hemolymph coagulation, melanotic encapsulation, induction of AMP synthesis and activation of cytokines (Cao et al., 2015;

Christophides et al., 2002; Krem and Di Cera, 2002; Zou et al., 2006, 2007). The exact biological function of the Clip-domain remains unclear; however, it may be responsible for mediating specific protein-protein interactions or for regulating cascades of SP activities. To date, Clip-SPs have only been identified in invertebrates. The powerful reverse genetic tools in *Drosophila* have been crucial to determine the functions of Clip-SPs and their organization in sequential cascades. In *Drosophila*, Clip-SPs are implicated in three fundamental biological mechanisms: **1)** the control of dorso-ventral patterning during embryonic development, **2)** the activation of the Toll-dependent response to microbial infections and **3)** the melanization cascade, which is mediated by the pro-Phenoloxydase (PPO).

Similarly to mammalian blood coagulation factors or complement cascades, insect proteases constitute a complex enzymatic system in the hemolymph preventing bleeding and limiting infection.

2. Structure and characteristics of Clip-SPs and Clip-SPHs in Arthropods

In the following sections, I will focus on the current knowledge of *Drosophila* Clip-SPs and Clip-SPHs as well as their involvement in the fruit fly physiology.

a. Domain organization and sequence of Clip-SPs

In *Drosophila melanogaster* genome, SPs and SPHs constitute the second largest family of genes (Ross et al., 2003). Of note, among the 147 SPs and 57 SPHs identified, 28 SPs and 14 SPHs carry regulatory Clip-domains (Piao et al., 2005; Veillard et al., 2015). Remarkably, the regulatory domains of 4 SPs (encoded by the genes *CG10232*; *CG12133*; *CG16710* and *CG8870*) and 1 SPH (encoded by the *CG3505* gene) were originally described as partial Clip-domains due to the lack of

the full protein sequences. The majority of Clip-SP folds carry a single Clip-domain, but several *Drosophila* genes encode proteins containing multiple Clip-domains: two Clip-domains in SP18, SPH58 and SPH121, three in SPH142 and five Clip-domains in SPH79 (Masquerade) (Ross et al., 2003).

In *Drosophila melanogaster*, these multiple Clip-domains are separated by a fairly long linker sequence with more than 30 residues (i.e., 71 residues in SP18; 38 residues in SPH58; 53 residues in SPH121). Contrarily, the lepidopteran Clip-SPs Prophenoloxidase-Activating Proteins (PAPs) from *Manduca sextra* have only between 1 and 7 residues connecting adjacent Clip-domains (Huang et al., 2007; Ross et al., 2003). Clip-domains are connected to the catalytic chymotrypsin fold by a 23 to 92 residues long linker containing at least one cysteine, which is involved in a disulfide linkage with another cysteine of the catalytic domain. The zymogens of Clip-SPs are converted into their catalytically active forms by a specific proteolytic cleavage at the amino-terminus of a residue corresponding to Arg15 of chymotrypsinogen. This proteolytic processing has been demonstrated biochemically for several Clip-SPs (Jiang et al., 1998; Lee et al., 1998; Muta et al., 1990; Satoh et al., 1999) and is presumed to also occur in the other members of the family. Owing to the disulfide bond, the N-terminal Clip-domain remains covalently attached to the catalytic proteinase domain after zymogen activation (Jiang and Kanost, 2000) **(Figure 6)**. Remarkably, the Clip-SPs never possess additional regulatory modules of other types (Veillard et al., 2015).

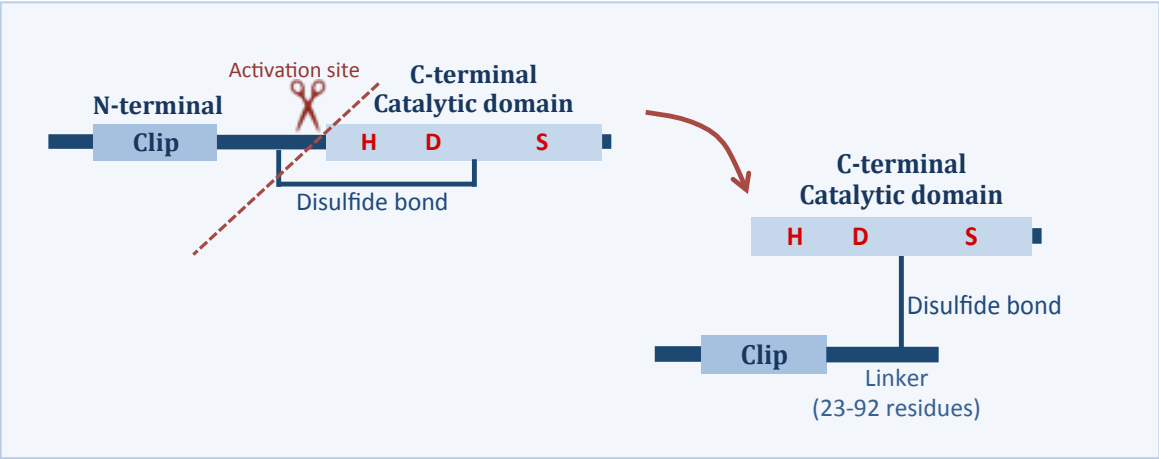


Figure 6: Domain architecture of Clip-Serine Proteases (Clip-SPs)

Clip-SPs contain one or more amino-terminal Clip-domains connected by a 23-92 residues long linker sequence to the carboxyl-terminal SP catalytic domain. The protease zymogen is activated by a specific cleavage at the activation site (corresponding to Arg15 of chymotrypsinogen), which is located at beginning of the catalytic domain. After this cleavage, the Clip-domain and the catalytic domain remain connected by an interchain disulfide bond. Clip-SPs have active sites with an intact catalytic triad His, Asp, Ser (H-D-S), marked in red in the figure. Clip-SPHs have a typical N-terminal Clip-domain a C-terminal SP-like domain in which one or more residues of the catalytic triad are lacking, disrupting the proteolytic activity.

b. Clip-domain properties: sequence and folding

The Clip-domain is a structural/regulatory unit harbored by several arthropod SPs involved in cascade pathways (Jiang and Kanost, 2000). It may be involved in shielding the catalytic site in a SP zymogen, interacting with associated proteins, or anchoring the SP to the surface of an invading organism. The sequence features of *Drosophila* Clip-domains were studied by several methods that revealed the presence of 46 Clip-domains in the identified Clip-SPs and SPHs (Ross et al., 2003). Clip-domains are usually 30 to 63 residues long with an

average of 45 residues. Commonly, there is a clear distinction between the Clip-domains from SPs and SPHs: the former are significantly longer. A typical Clip-domain in SPs is 42 to 55 residues long with an average of 50. On the other hand, most SPH Clip-domains span 30 to 45 residues with an average of 39. Clip-domains are characterized by six strictly conserved cysteine residues (Cys), which form 3 disulfide linkages (Cys1-Cys5; Cys2-Cys4; Cys3-Cys6) **(Figure 7)**. This structure has also been referred to as "disulfide knotted domains", but in order to avoid confusion with the "cystine knot" motif, the term Clip-domain was preferred (McDonald and Hendrickson, 1993). The Clip-domain was so named by Iwanaga's group because it could be drawn in the shape of a paper clip in a schematic form to show the disulfide linkages (Iwanaga et al., 1998; Jiang and Kanost, 2000). The overall sequence conservation between different Clip-domains is low (in the range of 20-45% identity), since the spacing between cysteine residues is highly variable, but the six Cys residues are strictly conserved. Of note, the sequence in the region from Cys1 to Cys3 is more conserved than that from Cys3 to Cys6 (Ross et al., 2003).

The Clip-domain is composed of a high portion of loops and a central four-stranded β-sheet stabilized by the three-disulfide bonds **(Figure 7)** (Kellenberger et al., 2011; Piao et al., 2005). The conserved three disulfide bonds knotting the loops and the β-strands together appear critical for the structural integrity of the central β-sheet that serves as the main framework of the Clip-domain structure (Piao et al., 2005). This four-stranded β-sheet structure is also observed in the lepidopteran PAP2 of *Manduca sexta* (*Ms*-PAP2) Clip-domain, while in the *Holotrichia diomphalia* Prophenoloxidase-Activating Factor II (*Hd*-PPAF-II), a Clip-SPH, the Clip-domain bears a three-stranded structure (Huang et al., 2007). The Cys1, Cys2, Cys5 and Cys6 residues are localized in central positions within the β-strands of these Clip-domains. Giving the preference for cysteine residues to be in β-strand conformation, the β-sheet structure is probably common to all Clip-domains (Bhattacharyya et al., 2004; Huang et al., 2007; Kellenberger et al., 2011). Generally, the main structural variations within Clip-domains reside between Cys3 and Cys4. Indeed, in the Clip-SPs *Drosophila melanogaster* Grass (*Dm*-Grass) and *Ms*-PAP2, this region embraces a helix-turn-

helix fold, whereas in the Clip-SPH *Hd*-PPAF-II the Cys3-Cys4 region forms a long loop containing a small stretch of β-strands (Piao et al., 2005). Independently of the structural differences in Clip-domains, usually the Cys3-Cys4 region in well-characterized Clip-domains constitutes a central hydrophobic cavity. The conservation of this characteristic motif suggests that it might constitute a protein interaction-binding site (Huang et al., 2007; Kellenberger et al., 2011; Piao et al., 2005, 2007).

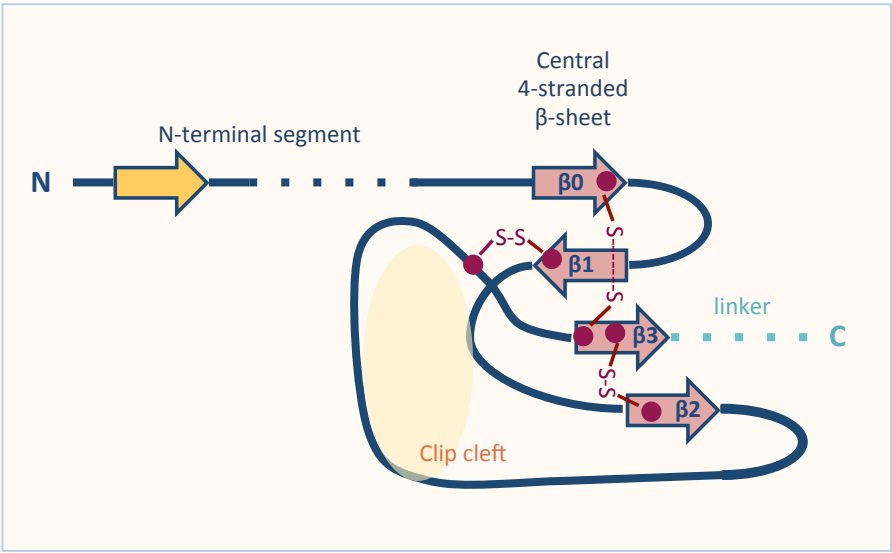


Figure 7: Schematic representation of the Clip-domain structure

Copyright (Piao et al., 2005)

The three-dimensional structures and functions of Clip-domains remain ambiguous; however, there has been considerable interest in enzymes containing these structures, because it seems likely that Clip-domains regulate Serine Protease activity and provide a site for interactions of proteinases with their activators, cofactors, and substrates.

Clip-domains are usually 30 to 63 residues long with an average of 45 residues. They are characterized by six strictly conserved cysteine residues (Cys), which form 3 disulfide linkages marked in fuschia in the figure. The Clip-domain is composed of a high portion of loops and a central four-stranded β-sheet stabilized by the three-disulfide bonds. The central β-sheet is composed of β0, β1, β2, and β3. Given that cysteine residues are localized in β-strand conformation, the β-sheet structure is probably common to all Clip-domain.

c. Structure of Clip-SPs

Despite the large number of Clip-SPs identified in Arthropods, the structures of only three Clip-SPs/SPHs have been determined: the first structure is that of *Hd*-PPAF-II, an inactive SPH acting as a cofactor of (Prophenoloxidase) PPO activating enzyme (Piao et al., 2005); the second structure is that of the catalytic domain of *Hd*-PPAF-I (Piao et al., 2007); and the third structure is that of the zymogenic form of *Dm*-Grass (Kellenberger et al., 2011). To date, the low number of structures resolved by crystallography limits the knowledge of the Clip-SPs folding. All three reported catalytic domains exhibit the characteristic fold of trypsin-like SPs arranged in two six-stranded β-sheets, which stack together (Bode and Schwager, 1975; Huber et al., 1974; Wang et al., 1985). The catalytic triad composed of the three conserved residues His57, Asp102 and Ser195 (bovine chymotrypsin numbering) locates in the cleft between the two sheets. Indeed, the superimposition of *Dm*-Grass onto chymotrypsin shows that 160 among 240 Cα (66%) of the model display equivalent positions in both molecules (Kellenberger et al., 2011). The remaining unaligned residues constitute the surface loops of the protease fold, named 30, 60, 70, 125, 140, 201, etc., according to the chymotrypsin numbering (Kraut, 1977). The transition from a zymogen to an active protease is associated with the formation, by a specific proteolytic cleavage, of a new N-terminus which becomes buried within the molecule and after a conformational change in the so-called "activation domain" (Kellenberger et al., 2011). In the *Dm*-Grass and *Hd*-PPAF-I zymogens, these loops adopt a closed conformation. In both zymogens, the conformation of the loop 140 blocks access to the catalytic space, thus preventing any residual activity in the zymogen (Kellenberger et al., 2011; Piao et al., 2007). During activation, the loop 140 undergoes conformational changes allowing access to the active site (Pasternak et al., 2001). This structural arrangement is also found in the trypsinogen zymogen. Indeed, the superimposition of *Dm*-Grass onto trypsin results in 58% topologically equivalent positions (135 Cα among 230) (Kellenberger et al., 2011). Both the *Dm*-Grass and *Hd*-PPAF-I 70-loops bind

calcium ions. This feature is also predicted for *Dm*-Easter SP based on a three-dimensional modeling study (Rose et al., 2003). It was shown that EDTA blocks the proteolytic power of *Hd*-PPAF-I on synthetic substrates, confirming the essential role of calcium binding for *Hd*-PPAF-I activity (Piao et al., 2007). Moreover, mutation in the calcium-binding loop of *Dm*-Easter results in a complete loss of function during *Drosophila* development (Rose et al., 2003). While in trypsin or coagulation factor Xa the calcium ion is involved in the active site (Rezaie and Esmon, 1994; Sipos and Merkel, 1970), in *Dm*-Grass and *Hd*-PPAF-I the 70-loop is distant from the active site suggesting that the calcium ion is not involved in the active site functioning, and that the loss of proteolytic activity of the arthropod SPs in absence of calcium is due to destabilization of their structure. Remarkably, compared to mammalian chymotrypsin-like SPs, *Dm*-Grass and *Hd*-PPAF-I have an additional 75-loop, which extends from the calcium binding 70-loop. The 75-loops stand near the zymogen activation site and may restrict its access to avoid unwanted cleavage (Kellenberger et al., 2011; Piao et al., 2007).

d. Active site organization, specificity and activation of Clip-SPs

Similarly to chymotrypsin-like proteases, X-ray structural studies reveal that the active site center of Clip-SPs corresponds to a catalytic triad composed of His57, Asp102 and Ser195 (chymotrypsin numbering) and responsible for the acyl transfer mechanism of catalysis (Blow, 1997; Hedstrom, 2002). The residues of the catalytic triad (Ser, His, Asp) are commonly found within three conserved regions: GDSGGP, TAAHC, DIAL respectively (Ross et al., 2003).

The active site of an enzyme performs the twofold function of binding the substrate and catalyzing the reaction. The efficiency of these actions determines the overall activity of the enzyme towards the particular substrate. In accordance with the nomenclature of Schechter and Berger, the active site of proteases corresponds to a succession of "subsites" or "pockets" (S1, S2, S3, S4 and S'1, S'2, S'3, S'4) located on both sides of the catalytic site. The specificity of SPs is usually

defined by the P1-S1 interaction, where P1-P'1 stand for the peptide residues surrounding the cleavage site on the substrate, and S1-S'1 designate the corresponding enzyme-binding sites (Schechter and Berger, 1967, 1968) **(Figure 8)**. The residues P on the substrate are designated P1, P2, etc. and P'1, P'2, etc. and counted from the point of cleavage towards the amino-and carboxyl-terminus respectively, and thus have the same numbering as the subsites they occupy.

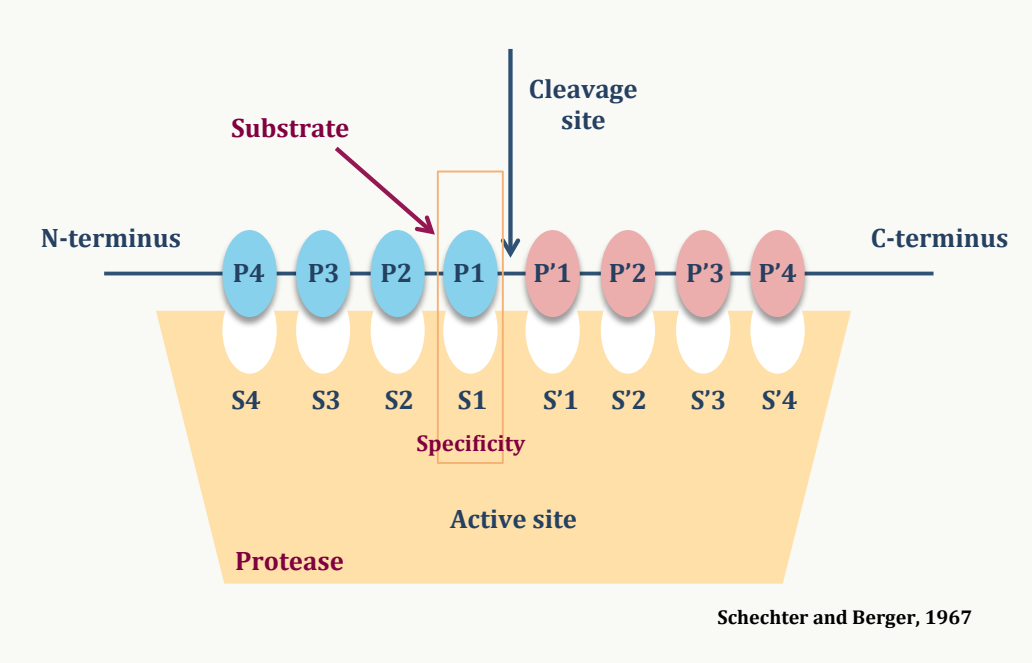


Figure 8: Schematic representation of the active site and the specificity of proteases

Based on the nomenclature of Schechter and Berger, the active site of a protease corresponds to a succession of "pockets" (S1, S2, S3, S4 and S'1, S'2, S'3, S'4). The specificity of SPs is determined by the P1-S1 interaction, where P1-P'1 are the peptide residues flanking the cleavage site on the substrate, and S1 and S'1 stand for the corresponding enzyme-binding sites.

The blue arrow designates the sessile peptide bond between P1 and P'1 residues. SPs have a specific recognition site where the peptide bond is cleaved. As an example, trypsin only cleaves at lysine (Lys) or arginine (Arg) residues, but does not matter which amino acid is located at position P'1 (carboxyl-terminal of the cleavage site). Another example is trombin which cleaves if an Arg is found in position P1, but not if an aspartic acid (Asp) or a glutamic acid (Glu) residue is present in position P1' at the same time.

The S1 site of chymotrypsin-like fold of SPs corresponds to a pocket adjacent to Ser195 and formed by residues 189-192, 214-216 and 224-228. The protease-substrate specificity is mainly determined by the 3 residues at positions 189, 216 and 226 (Czapinska and Otlewski, 1999; Perona and Craik, 1995, 1997). In chymotrypsin, the combination of Ser189, Gly216 and Gly226 creates a deep hydrophobic pocket that accepts large aromatic residues preferentially. In trypsin-like SPs, the Asp189, Gly216 and Gly226 combination forms a negatively charged S1 site that determines specificity for substrates containing Arg or Lys at the P1 site (Huber et al., 1974). By these criteria, Clip-SPs in *Drosophila* should have trypsin-like specificities, since their S1 pockets carry the characteristic Asp189, Gly216 and Gly226 residues (Ross et al., 2003). Non-Clip SPs in *Drosophila* may have different proteolytic specificities. Of note, ModSP, which participates in the extracellular cascade upstream of the Toll receptor, as well as Gastrulation-defective (Gd), which is implicated in the embryonic development, both bear the characteristic Ser189, Gly216 and Gly226 residues in their S1 pocket, providing them a chymotrypsin-like activity.

The activation of Clip-SPs relies on a proteolytic cleavage at the N-terminal extremity of their catalytic domain. This cleavage allows the liberation of a consensus sequence I/V-V-G-G found at the N-terminal extremity of the majority of SPs. Upon activation, this motif becomes buried within the protease leading to a conformational change in the so-called “activation domain” allowing the catalytic domain to become functionally active (Hedstrom, 2002; Kellenberger et al., 2011; Veillard et al., 2015).

e. Biochemical, structural and physiological properties of Clip-SPHs

Conversly to SPs, SPHs are enzymatically inactive. Most often the active site serine residue is changed to glycine in the catalytic triad of SPHs in arthropods (Kanost and Jiang, 2015). To date, the crystallographic structure of only one SPH

has been successfully resolved: it is that of the beetle *Holotrichia diomphalia Hd*-PPAF-II (Piao et al., 2005). The SP-like catalytic domain of *Hd*-PPAF-II shares relatively high sequence similarity with chymotrypsin, with two six-stranded β-barrels stacked together. *Hd*-PPAF-II is devoid of a catalytic activity because a glycine (Gly) residue is found at the position of the invariant Ser nucleophile in the catalytic SPs (Ser353), disrupting the His-Asp-Ser catalytic triad present in SPs of the Chymotrypsin family. The SP-like catalytic domain contains two clefts, one of which corresponds to the defective active site cleft containing the Gly-His-Asp triad (instead of the Ser-His-Asp) (Piao et al., 2005).

In Clip-SPs, the Clip-domain is connected to the catalytic domain by a flexible linker that is sensitive to proteolysis, whereas this linker is absent in Clip-SPHs.

The physiological functions of *Drosophila* Clip-SPHs are poorly understood, although they have been implicated in different arthropod immune responses, in the horseshoe crab (Kawabata et al., 1996), *Manduca sexta* (Felföldi et al., 2011) and *Anopheles gambiae* (Dimopoulos et al., 1997; Povelones et al., 2013). In *D. melanogaster* as well, Clip-SPHs have been reported to exert functions during the innate immune response: (SPH93) (Gordon et al., 2008) and (Spheroide) (Patrnogic and Leclerc, 2017). In addition, Clip-SPHs have also been shown to participate in somatic muscle attachment (Masquerade) (Murugasu-Oei et al., 1995), in the regulation of hemocyte proliferation (CG4793) (Bina et al., 2010) or the morphogenesis of imaginal discs (Scarface, CG11066) (Rousset et al., 2010). Due to their lack of proteolytic activity, the Clip-SPHs probably act as cofactors, or binding partners, for active Clip-SPs.

In this regard, the best-characterized molecular mechanism involving a Clip-SPH is PPO activation in the beetle *H. diomphalia*. A two-step mechanism for PPO activation was proposed, in which the full-length (79 kDa) PPO is first cleaved by the Clip-SP *Hd*-PPAF-I into a 76-kDa form (ProPO76), which does not exhibit any PO activity, and subsequently converted into the 66-kDa active form (PO66) by *Hd*-PPAF-I in the presence of the Clip-SPH *Hd*-PPAF-II (Kim et al., 2002; Kwon et al., 2000). Accordingly, the Clip-SPH *Hd*-PPAF-II deprived of enzymatic activity, acts as an essential cofactor for the activation of PO by the Clip-SP *Hd*-PPAF-I.

3. Functions of *Drosophila* Clip-Serine Proteases (Clip-SPs)

a. Clip-SPs in the Establishment of the dorso-ventral axis during embryo development

Clip-SPs were first described for their role during *Drosophila* embryonic development, where they control the initiation of the dorso-ventral polarity (LeMosy et al., 1999). The basic body plan of *Drosophila* is determined during the blastoderm stage (Jaeger et al., 2012; St Johnston and Nüsslein-Volhard, 1992). During blastoderm formation, the dorso-ventral axis is specified through a morphogenetic nuclear gradient of the NF-κB transcription factor Dorsal (Lemaitre et al., 1995b; Steward et al., 1988). Dorsal is uniformly translated in all embryonic cells however; it is translocated into nuclei only in ventral embryonic regions providing the ventral identity of the embryo. An extracellular proteolytic cascade leading to the activation of the Toll receptor on the surface of the ventral embryonic cells regulates the nuclear translocation of Dorsal. Toll responds to this ventrally localized extracellular signal in the perivitelline space between the oocyte membrane and the follicle cells (Hashimoto et al., 1988; Moussian and Roth, 2005; Schneider et al., 1991). Genetic approaches showed that the proteolytic cascade that triggers this extracellular signal is formed by the SPs Nudel (Ndl), Gastrulation-defective (Gd), Snake (Snk) and Easter (Ea) **(Figure 9)**, culminating with the proteolytic activation of the cytokine Spz (Chasan et al., 1992; Han et al., 2000; Hong and Hashimoto, 1995; Konrad et al., 1998), which binds the Toll receptor (DeLotto and DeLotto, 1998; Morisato and Anderson, 1994; Schneider et al., 1994; Smith and DeLotto, 1994; Smith et al., 1994). During development, the Toll receptor is equally distributed throughout the egg membrane, while Spz and the SPs of the extracellular cascade are secreted into the perivitelline space, with the exception of Ndl (Hashimoto et al., 1988, 1991). Ventral restriction of active Spz formation depends on the sulfotransferase Pipe (Hashimoto et al., 1991; Stein and Stevens, 1991), which is expressed in ventral cells of the follicular epithelium surrounding the developing oocyte (Sen et al., 1998), while expression in dorsal follicle cells is suppressed by the transcription

factor Mirror (Jordan et al., 2000). Deletion of the *pipe* gene blocks the generation of activated Spz and thus the nuclear translocation of Dorsal (Morisato and Anderson, 1994). Pipe was shown to modify the ventral vitelline envelope by sulfating the proteins at its surface to produce a ventral cue (Zhang et al., 2009), which promotes the proteolysis steps in the perivitelline space **(Figure 9)**. It was demonstrated that the proteolysis of Ea by Snk is a Pipe-dependent step and a ventrally restricted event in the proteolytic cascade (Cho et al., 2010).

Both, (*snk*) and (*ea*) genes encodes Clip-SPs, while (*gd*) encodes a larger SP that shares homology with the mammalian complement factors C2 and B (DeLotto, 2001). Dissing et al. demonstrated the sequential activation of Gd, Snk, Ea and Spz *in vitro* using a heterologous co-expression system (Dissing et al., 2001). Ndl is a large (320 kDa) multi-domain protein resembling an extracellular matrix protein that carries a central SP domain, several LDLa domains, and a C-terminal serine/threonine-rich domain. Unlike the other three SPs in the cascade, which are secreted by the germline cells, Ndl is secreted by the somatic follicle cells and appears not to circulate in the perivitelline space (Hong and Hashimoto, 1995, 1996). Processing of Gd is blocked in flies carrying mutations in Ndl (Cho et al., 2010) however; Gd can be auto-proteolytically activated in presence of Snk and in the absence of Ndl when expressed in a heterologous system (LeMosy et al., 2001). Beside its enzymatic activity, Gd is also implied in the regulation of Ea activation (Cho et al., 2010). Indeed, at high local concentration, Gd interacts with both Snk and Ea on the surface of the ventral vitelline space (Cho et al., 2012). These interactions are essential for Ea activation by Snk and explain the spatial regulation of the production of activated Spz.

b. Clip-SPs in the melanization pathway

The melanization reaction is an immediate immune response against invading pathogens in *Drosophila* (Cerenius and Söderhäll, 2004; Cerenius et al., 2008;

Tang, 2009). Melanization is visible by the blackening of a wound site resulting from the synthesis and deposition of melanin. The pigment appears on the surface of the cuticle as well as on and near the surfaces of microorganisms that have invaded the hemocoel of the host (Christensen et al., 2005). In addition to being important for wound healing, melanin can encapsulate the pathogens and the reaction intermediates, such as reactive oxygen species (ROS), generated during melanin production appear to be directly harmful to microbes (Nappi and Christensen, 2005). During this pathway, the enzyme Phenoloxidase (PO), with tyrosinase-like activity, catalyzes the oxidation of phenols to quinones, which polymerize non-enzymatically to form melanin. Quinones may also be involved in the production of cytotoxic superoxides and hydroxyl radicals that could help to kill the intruders (Gillespie et al., 1997; Zhao et al., 2007, 2011). In *Drosophila* and other Arthropods, PO is produced and released into the hemolymph as an inactive zymogen called prophenoloxidase (PPO).

Biochemical studies in larger insect species, such as the silkworm *Bombyx mori* (Satoh et al., 1999), the tobacco hornworm *Manduca sexta* (Kanost et al., 2004), or the beetle *Holotrichia diomphalia* (Lee et al., 1998) have identified Clip-SPs proteases that cleave and activate PPO at the end of a proteolytic cascade: the PPO-Activating Enzymes (PPAEs) also known as PPO-Activating Factors (PPAFs), or PPO-Activating Proteins (PAPs) (Jiang et al., 1998; Lee et al., 2002). In several insect species such as *Manduca sexta* and the mealworm *Tenebrio molitor*, activation of the melanization pathway during infection depends on the detection of pathogens by circulating PRRs such as C-type lectins (Yu et al., 1999), GNBPs (Jiang et al., 1998; Matskevich et al., 2010; Yu et al., 2002), or PGRPs (Park et al., 2006; Schmidt et al., 2008).

Despite extensive genetic studies, the melanization reaction remains poorly characterized in *Drosophila*. But PRRs seem to be involved in the triggering of the melanization. For instance, PGRP-LE was shown to induce melanization in *Drosophila* in response to Gram-negative bacterial infection (Takehana et al., 2004).

The *Drosophila* genome encodes three PPOs. At the larval stage, PPO1 and PPO2

are expressed in crystal cell hemocytes. Upon injury, crystal cells rupture and release PPOs into the hemolymph where they are activated by Clip-SPs (Bidla et al., 2007). In adult flies, PPO1 and PPO2 are found circulating in the hemolymph, but their synthesis sites have yet to be identified (Honti et al., 2014). The third enzyme, PPO3 is predominantly found in the lamellocytes. It was demonstrated that uncleaved *Drosophila* PPO3, but not PPO1 or 2, is enzymatically active in its zymogen form (Nam et al., 2008).

Three immune inducible Clip-SPs with trypsin-like specificity and implicated in the melanization pathway have been identified in *Drosophila*: Hayan, Melanization Protease 2 (MP2) (also called PAE1 or Sp7) and MP1 (Castillejo-López and Häcker, 2005; Leclerc et al., 2006; Tang et al., 2006). Similarly to PPAFs of larger insects, MP2, MP1 and Hayan participate in the cleavage and the activation of PPOs **(Figure 9)**.

MP2 activates MP1 upon PRR-mediated recognition of microbial motifs (An et al., 2013; Tang et al., 2006). Overexpression of either MP1 or MP2 results in constitutive melanization and semi-lethality, while knock down of MP1 or MP2 compromises POs activation and melanization upon immune challenge (Castillejo-López and Häcker, 2005; Leclerc et al., 2006). It was reported by Tang et al. that MP1 is required to activate melanization in response to both bacterial and fungal infection, whereas MP2 is mainly involved during fungal infection. Pathogenic bacteria and fungi may therefore trigger two different melanization cascades that use MP1 as a common downstream activator of PO. In MP2 mutant flies, the cleavage of PPO is blocked after infection. MP1 was shown to act downstream of MP2, making it a potential PPAE that directly cleaves and activates PPO. In transfected *Drosophila* S2 cells, pre-activated MP2 failed to cleave the MP1 zymogen suggesting that at least one other protease functions in between these two SPs (Tang et al., 2006).

Moreover, melanization reaction, although not essential for survival following microbial infection, plays a critical role in enhancing the effectiveness of other immune responses in *Drosophila*. The remaining Clip-SP Hayan is implicated in the melanization mechanism during systemic wound response showing that

activation of Hayan is not dependent on PRR (the response to septic or sterile injuries) (Nam et al., 2012). Interestingly, neither MP1 nor MP2 are required for this mechanism, suggesting the existence of independent proteolytic cascades leading to PPO activation.

c. Clip-SPs in the Toll pathway during immune responses of *Drosophila*

The hallmark of the humoral response in *Drosophila* is the induction, following microbial challenge, of a series of AMPs in the fat body followed by the secretion of these peptides into the hemolymph of the insect (Hoffmann, 2007; Hoffmann and Reichhart, 2002; Lemaitre and Hoffmann, 2007). As mentioned previously **(section IV)**, two main signaling pathways are implicated in the activation of AMP gene expression: the Toll and the IMD pathways (Imler et al., 2004). To date, four SPs have been clearly identified in the Toll cascade: one non-Clip SP, Modular Serine Protease (ModSP), and three Clip-domain SPs Grass, Psh and the terminal SP Spaezle Processing Enzyme (SPE) **(Figure 9)**. The activation of the danger arm of the Toll pathway by microbial virulence factors depends on the Clip-SP Psh, which belongs to the chymotrypsin S1 family **(described previously)**.

The Clip-SP SPE was identified by homology with Ea (Jang et al., 2006; Mulinari Shai et al., 2006) **(Cascade implicating Ea described in the paragraph a of this section)**. The non-Clip ModSP was identified by homology to the *Tenebrio molitor* modular SP, *Tm*-MSP and the tobacco hornworm *Manduca sexta* SP Hemolymph Proteinase 14 (HP14) (Wang and Jiang, 2006). Similarly to its homologs, ModSP is a large multi-domain SP carrying 4 LDLa domains and a Complement Control Protein (CCP) module at its N-terminus (Buchon et al., 2009). It was demonstrated that in *T. molitor* and *M. sexta*, binding of microbial peptidoglycans to the PGRP-SA/ GNBP-1 complex triggers activation of Tm-MSP and HP14 respectively by an auto-processing mechanism (Kan et al., 2008; Kim et al., 2008). *In vitro* data show that ModSP is unstable and quickly undergoes an

auto-proteolytic activation. Thus, it was assumed that ModSP is auto-activated similarly to *Tm*-MSP by integrating the signal generated by the binding of the microbial ligands to their circulating cognate receptors (Buchon et al., 2009). Interestingly, this mechanism resembles the complement activation in mammals, in which recognition of carbohydrates by the circulating MBL leads to the auto-activation of MASPs, which are also modular serine proteases containing a CCP domain (Dobó et al., 2014; Duncan et al., 2008).

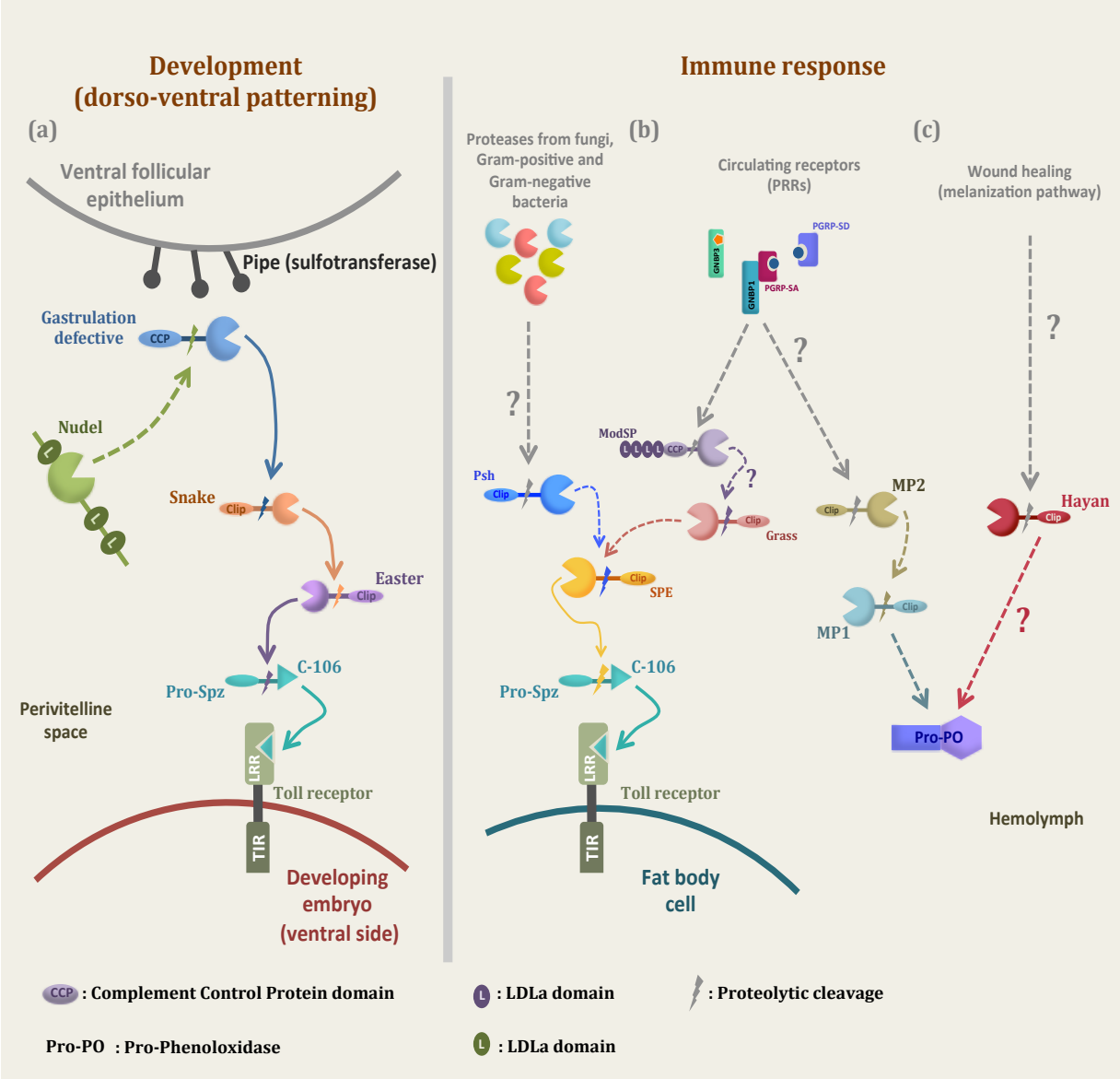


Figure 9: Clip-domain Serine-Proteases (Clip-SPs) extracellular cascades in *Drosophila melanogaster*

(Described on the next page)

In *Drosophila*, Clip-SPs form extracellular proteolytic cascades participating in the establishment of the dorso-ventral patterning during embryonic development (a) and in the humoral immune responses of the adult flies (Toll pathway (b) and melanization cascade (c)). Full arrows are used when the interaction has been demonstrated by genetic studies and *in vitro* reconstitution, whereas dashed arrows represent links observed only by genetic analysis. Question marks indicate that the mechanism linking two components of the pathway is not elucidated or that an unidentified component is probably missing. These pathways are described in details in the main text.

4. Regulation of Clip-SPs in *Drosophila*

a. Negative regulation: Serpins (SERine Protease INhibitorS)

In biological systems, proteases and protease inhibitors often exist as pairs in order to maintain homeostasis (Jiang and Kanost, 2000). Serine protease inhibitors, of the serpin superfamily share a conserved fold and function mostly as protease inhibitors. They are extensively studied in mammals where they are well known as regulators of hemostasis, thrombolysis, inflammation or complement activation (Silverman et al., 2001). The biological importance of serpins is highlighted by serpin dysfunction diseases, such as thrombosis caused by a deficiency in antithrombin (Ligoxygakis et al., 2003). The serpin superfamily is represented in all branches of life, within Eukaryotes and Prokaryotes, and constitutes the largest class of peptidase inhibitors, with over 1500 members identified to date (Huntington, 2011; Law et al., 2006).

Serpins are relatively large molecules (about 330-500 amino acids) in comparison with other protease inhibitors such as basic pancreatic trypsin inhibitor (BPTI, which is about 60 amino acids) (Rühlmann et al., 1973). Serpins act as suicide substrates and are cleaved by their target protease, forming an

essentially irreversible, covalently linked 1:1 serpin/protease complex (Gettins, 2002; Ligoxygakis et al., 2003).

Serpins fold into a conserved secondary structure and adopt in their native state, a metastable conformation allowing them to employ a unique irreversible suicide-substrate inhibitory mechanism (Silverman et al., 2001). The serpin fold comprises 3 β-sheets A, B and C, with 8 or 9 α-helices (Reichhart et al., 2011). Inhibitory serpins expose a C-terminal reactive-center loop (RCL) acting as a bait for the target protease (Ellisdon et al., 2014). Upon interaction with their targets, serpins undergo major structural rearrangements that involve alternative conformations for the RCL, β-sheet A, and the attached strand 1 of β-sheet C (Stein and Carrell, 1995). Accordingly, serpins ensure tight physiological specificity even when confronted by a group of proteases whose cleavage specificity is similar (Whisstock et al., 2010).

When a SP cleaves the scissile peptide bond between residues P1 and P'1 within the RCL of the serpin, it is subsequently trapped to the backbone carbonyl of the P1 residue via a covalent ester linkage with the Ser195. The SP is then translocated to the other side of the serpin and its active site is distorted, which renders the protease inactive (Silverman et al., 2001). Serpin-SP complexes have an extremely short half-life as members of the low-density lipoprotein receptor (LDL) family are in charge of eliminating them from the circulation (Kasza et al., 1997; Storm et al., 1997).

Remarkably, genome surveys based on the presence or the absence of RCL indicate that up to one-third of a species serpin arsenal may have non-inhibitory features (Silverman et al., 2010). Non-inhibitory serpins appear to derive from inhibitory serpins (Garrett et al., 2009). In *Drosophila melanogaster*, Accessory gland peptide 76A (Acp76A), also known as Spn76A, is a putative non-inhibitory serpin that is expressed at high levels in the male accessory gland and transferred to the female genital tract during mating (Coleman et al., 1995; Reichhart et al., 2011). In humans, several serpins have been reported to function as hormone transporters (Pemberton et al., 1988), others as molecular chaperones (Ishida and Nagata, 2011; Nagata, 1996) or tumor suppressors (Zou et al., 1994). Investigations aiming at elucidating the partners and roles of non-inhibitory serpins will represent a major challenge in the field.

In *Drosophila*, several serpins have been reported to regulate the Clip-SP proteolytic cascades induced during immune responses and embryonic development. 29 serpin genes are present in *Drosophila* genome. One of these genes, *Spn42Da*, encodes eight protein isoforms (Ellisdon et al., 2014). Based on the RCL region, 24 serpins among the expressed 36 are considered as active inhibitors. 17 out of these active 24 possess a secretion signal peptide indicating that they act at the extracellular level and represent potential candidates for inhibiting extracellular SPs (Reichhart, 2005; Reichhart et al., 2011).

i. Regulation of the development extracellular cascade

Spn27A is implicated during early *Drosophila* embryogenesis where it participates in the orientation of the dorso-ventral axis (Hashimoto et al., 2003). **(For the development pathway, see figure 9)**. Female flies deficient for Spn27A produce embryos that show constant high levels of Toll signaling due to a uniform activation of the dorso-ventral pathway proteases around the embryonic circumference. These embryos lack dorso-ventral polarity and exhibit ventral structures at the expense of lateral and dorsal fates (Ligoxygakis et al., 2003).

ii. Regulation of the melanization pathway

The melanization pathway needs to be tightly regulated and locally restricted, because excessive PPO activation could cause fatal damage to the host (Zhao et al., 2005; Zhu et al., 2003). Genetic studies have showed an implication of serpins in the regulation of the melanization cascade. **(For the melanization pathway, see figure 9)**.

Accordingly, Spn27A regulates the melanization reaction in the hemolymph (De Gregorio et al., 2002a; Ligoxygakis et al., 2002a). Loss-of-function mutations in

the *Spn27A* gene result in a high level of spontaneous melanization and constitutively elevated PO activity, while overexpression of Spn27A suppresses PO activation following microbial immune challenge. Recombinant Spn27A was shown to inhibit *in vitro* the coleopteran PPAE from *Holotrichia diomphalia* (De Gregorio et al., 2002b); however, the endogenous target of Spn27A in *Drosophila* has remained unknown. MP1 and MP2 have been proposed as potential target proteases. Interestingly, Spn27A also regulates Hayan-mediated melanization during wound healing despite the lack of involvement of MP1 or MP2 in the response to systemic injuries (Nam et al., 2012).

In addition to Spn27A, at least two other serpins participate in the regulation of melanization events: Spn28D and Spn77Ba. Spn28D, an injury-induced serpin, was shown to regulate hemolymph PO activity in both larvae and adult flies at a different level than Spn27A. It was demonstrated that knock down of the *CG7219* gene, encoding Spn28D, causes and over-reactive PO activity upon septic injury, leading to extensive melanization in various tissues, especially those in direct contact with the external environment. Moreover, it was suggested that Spn28D prevents spontaneous activation of PO after its release in the hemolymph (Scherfer et al., 2008).

Melanization in *Drosophila* trachea can be activated by the presence of microorganisms but is normally blocked by Spn77Ba (Tang et al., 2008). Spn77Ba regulates the proteolytic cascade involving the MP1 and MP2 proteases. Deletion of Spn77Ba results in constitutive melanization in the respiratory system and causes lethality, which is rescued by the knock down of MP1 or MP2.

iii. Regulation of the extracellular cascade of the immune Toll pathway

The first *Drosophila* serpin identified in the control of the immune response is Necrotic (Nec), encoded by the *Spn43Ac* gene. Nec negatively regulates the Toll signaling pathway. Loss-of-function *nec* adult mutants develop black necrotic

spots along the body and the leg joints and flies die shortly after hatching. They also accumulate cleaved active Spz leading to a constitutive expression of *drosomycin* (Levashina et al., 1999). The lethal phenotype related to the *nec* mutation was used later in a suppressor screen and allowed the identification of Psh as a crucial Clip-SP for the Toll pathway activation (Ligoxygakis et al., 2002b). Psh has been suggested to be the Nec target Clip-SP; however, no direct interaction has been established so far. Nec carries a long N-terminal extension that is cleaved *in vivo* following microbial challenge (Pelte et al., 2006). Recombinant Nec lacking this N-terminal extremity exhibits a broad specificity and inhibited *in vitro* a wide range of proteases such as elastase, thrombin and chymotrypsin-like SPs, but failed to regulate tryspin or kallikrein (Robertson et al., 2003). Similarly to mammalian serpins, Nec is uptaken from the hemolymph by the lipophorin receptor-1 that belongs the LDL receptor family (Soukup et al., 2009). **(For the immune Toll pathway, see figures 3, 9)**.

b. Role of invading pathogens in Clip-SPs regulation

Clip-SPs are fundamental components of the immune system of insects. Thus, they can be mainly targeted by virulence factors produced by the invading pathogens. To date, no specific examples have been reported in *Drosophila*, while examples of Clip-SPs regulation have been identified in other insect species (Veillard et al., 2015). An example is the entomophathogenic nematode *Steinernema carpocapsae*, which is widely used for the control of insect pests due to its virulence and selectivity (Lacey and Shapiro-Ilan, 2008). *S. carpocapsae* releases a serpin-like inhibitor (Sc-SRP-6), which forms a stable complex with a tryspine-like enzyme from the Lepidoptera *Galleria mellonella*, preventing the formation of hard melanized clots following PPO activation (Toubarro et al., 2013).

A second example is that of parasitoid wasps, which inject maternal factors into the host's general cavity, during a process called oviposition, to suppress the

immune system and ensure successful development of their progeny in the host. These factors include viruses, virus-like particles such as polydnaviruses (PDVs), or venom fluids (Edson et al., 1981). Successful development of the parasitoid egg within the host depends on the presence of the virus, which acts to suppress the host's immune response (encapsulation) towards the egg. *Microplitis demolitor* bracovirus (MdBV) carried by the wasp *M. demolitor* encodes three proteins (Egf1.0, Egf1.5 and Egf0.4) characterized by a shared cysteine-rich motif with similarities to the trypsin inhibitor-like domain of small serine protease inhibitors (smapins) (Beck and Strand, 2007). It has been demonstrated that both Egf1.0 and Egf1.5 inhibit the processing and the amidolytic activity of PAP1 and PAP3 from *M.* sexta, and thus block PPO activation and melanization (Lu et al., 2010).

Venom proteins have been shown to play crucial roles in host regulation. The venom of the Hymenopteran parasitoid wasp *Cotesia rubecula* contains a small SPH-like venom protein of 50-KDa, designated as Vn4.6. Similarly to other SPHs, Vn4.6 consists of an N-terminal Clip-domain and a SP-like domain. This venom protein blocks melanization in the hemolymph of its host by interfering with the activation of the host's PPO (Asgari et al., 2003; Zhang et al., 2004).

VI. Manuscript Introduction

In general, bacteria and fungi secrete a wide spectrum of proteases, ranging from non-specific enzymes able to degrade any component of the immune response, to proteases that are extremely specific to their host's immune target (Frees et al., 2013; Lebrun et al., 2009; Monod et al., 2002). In *Drosophila melanogaster*, sensing of danger signals is suggested to take place in the hemolymph by the Clip-SP "Psh" upstream of the Toll receptor. Because *Drosophila* Clip-SPs are organized in cascades, the activation site of one Clip-SP should match the specificity of the upstream protease. Thus, a majority of Clip-SPs present an Arginyl or Lysyl residue in P1 position of the activation site (Schechter and Berger nomenclature, 1967) to be processed by proteases with trypsin-like specificity (Ross et al., 2003).

Remarkably, Psh should be theoretically insensitive to activation by most proteases and endogenous Clip-SPs because of an unusual Histidine residue in P1 position, which is accepted by very few proteases active site. This structural particularity is in complete opposition with a role for Psh in the detection of a broad range of proteases secreted by invading microbes.

Accordingly, the fungal protease PR1 was shown to activate the Toll pathway in a Psh-dependent manner when expressed in adult flies (Gottar et al., 2006). Moreover, purified proteases from *Aspergillus oryzae* or *Bacillus subtilis* are able to activate the Toll pathway via Psh when injected to the body cavity of the flies (El Chamy et al., 2008).

The specificity and the precision of the cleavage needed to activate the zymogen of Psh, is difficult to reconcile with the broad structural diversity of secreted microbial proteases, led us to investigate how danger signals can engage the *Drosophila* immune system and to decipher the molecular mechanism by which Psh is activated upon detection of an abnormal proteolytic activity in the hemolymph.

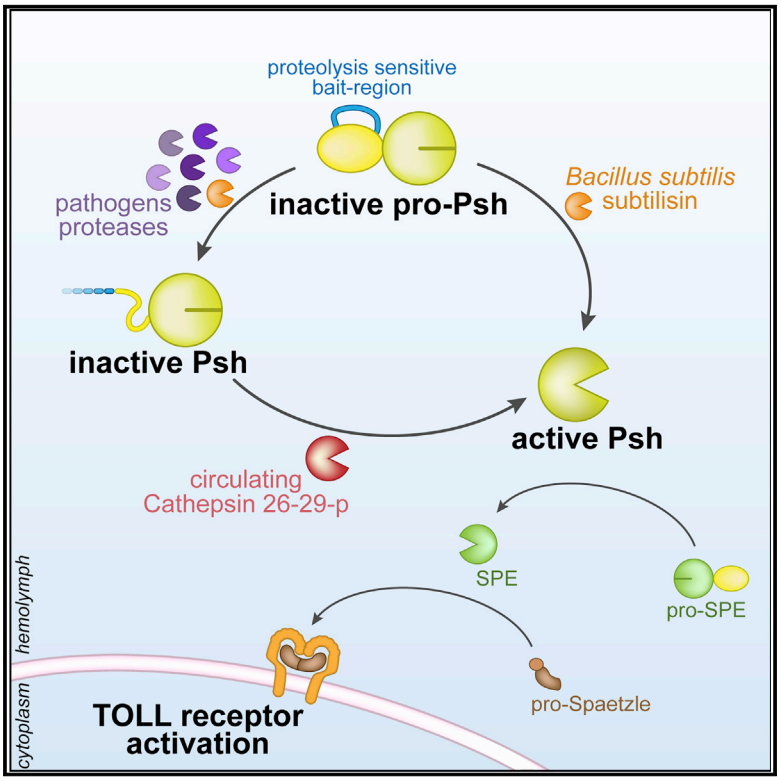
VII. Manuscript

Article

Molecular Cell

The Circulating Protease Persephone Is an Immune Sensor for Microbial Proteolytic Activities Upstream of the *Drosophila* Toll Pathway

Graphical Abstract



Highlights

- All pathogen-secreted proteases activate the danger-sensing arm of the Toll pathway
- The protease Persephone is the immune sensor for microbial proteolytic activities
- A sensitive region in Persephone zymogen functions as a bait for exogenous proteases
- Bait-region hydrolysis primes maturation of Persephone by the host cathepsin 26-29-p

Authors

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In Brief

Innate immune systems are activated by microbial molecular patterns or pathogen functional features. Issa et al. show that the *Drosophila* Toll pathway senses pathogen proteases through a hydrolysis-sensitive region localized in the Persephone pro-domain. Cleavage of this bait region primes maturation of Persephone and activation of the pathway by the host cathepsin 26-29-p.

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The Circulating Protease Persephone Is an Immune Sensor for Microbial Proteolytic Activities Upstream of the *Drosophila* Toll Pathway

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SUMMARY

Microbial or endogenous molecular patterns as well as pathogen functional features can activate innate immune systems. Whereas detection of infection by pattern recognition receptors has been investigated in details, sensing of virulence factors activities remains less characterized. In *Drosophila*, genetic evidences indicate that the serine protease Persephone belongs to a danger pathway activated by abnormal proteolytic activities to induce Toll signaling. However, neither the activation mechanism of this pathway nor its specificity has been determined. Here, we identify a unique region in the pro-domain of Persephone that functions as bait for exogenous proteases independently of their origin, type, or specificity. Cleavage in this bait region constitutes the first step of a sequential activation and licenses the subsequent maturation of Persephone to the endogenous cysteine cathepsin 26-29-p. Our results establish Persephone itself as an immune receptor able to sense a broad range of microbes through virulence factor activities rather than molecular patterns.

INTRODUCTION

The metazoan innate immune system has developed numerous strategies to control fungal and bacterial infections, ranging from physical barriers to a sophisticated array of molecules and cells that function to suppress or prevent microbial invasion. Initial recognition of microbial invaders is mediated by a set of germline-encoded pattern recognition receptors (PRRs) that sense highly conserved pathogen-associated molecular patterns (PAMPs) not present in the host (Medzhitov and Janeway, 2002). Engagement of PRRs activates intracellular signaling pathways leading to the expression of pro-inflammatory cytokines, chemokines, and soluble antibacterial effector molecules. PRRs also recognize endogenous molecules or damage-associated molecular patterns (DAMPs) released during tissue or cellular damage resulting from infection or tissue injury (Bianchi, 2007). Innate immune system can also be activated by pathogen functional features acting as danger signals such as toxins or enzymatic activity of virulence factors. Whereas the activation of PRRs by microbial or endogenous molecular patterns has been characterized in structural detail, the sensing of danger signals remains less well understood (Yin et al., 2015).

In *Drosophila melanogaster*, two evolutionarily conserved signaling pathways, Toll and immune deficiency (IMD), control the expression of anti-microbial peptides (AMPs) following immune challenge (Hoffmann, 2003). The IMD pathway, activated by diaminopimelic acid-containing peptidoglycan (DAP-type PGN) common to most Gram-negative bacteria, regulates expression of a set of AMPs, among which is Diptericin, via the NF-κB transcription factor Relish (Kleino and Silverman, 2014). On the other hand, the Toll pathway is activated by lysine-containing peptidoglycan (Lys-type PGN) found in Gram-positive bacteria and by β-glucans characteristic of fungal cell walls and activates a different set of AMPs, including the antifungal peptide Drosomycin (Drs), through dorsal-related immune factor (DIF), another NF-κB-like transcription factor (Valanne et al., 2011). Of note, sensing of these PAMPs occurs upstream of the receptor Toll, which functions as a receptor for the cytokine Spaetzle (Spz) in *Drosophila* (Levashina et al., 1999; Weber et al., 2003). Circulating PRRs, e.g., peptidoglycan recognition protein (PGRP)-SA for Lys-PGN or glucan-binding protein (GNBP) 3 for β-1-3 glucans, sense infection in the hemolymph and activate a serine protease referred to as modular serine protease (ModSP). Activation of ModSP triggers the sequential activation of the Clip-serine proteases (Clip-SPs) Grass and Spaetzle-processing enzyme (SPE) (Buchon et al., 2009; El Chamy et al., 2008; Gobert et al., 2003; Gottar et al., 2006). The latter processes the Spz precursor to generate an active Toll ligand. Of note, a second cascade, the so-called danger signal cascade, can independently activate SPE and the Toll receptor. Rather than PRRs, it is dependent on the Clip-SP Persephone (Psh) and has been shown to be activated by some bacterial or fungal proteases (El Chamy et al., 2008; Gottar et al., 2006; Ligoxygakis et al., 2002). However, neither the activation mechanism of this arm of the Toll pathway nor its specificity has been determined. Microbial proteases play a central role in the host colonization

and in the control of the immune system. Various examples have emerged across species showing that during close host-pathogen co-evolution, immune systems developed the mean to sense this danger (Chavarría-Smith et al., 2016; Cheng et al., 2015; de Zoete et al., 2011; LaRock et al., 2016; Turk, 2007). However, due to the high variety of protease enzymatic specificities, such systems are able to detect only a limited number of proteases.

Clip-SPs such as Psh belong to the chymotrypsin family and are expressed and secreted as inactive zymogens with a regulatory N-terminal pro-domain or "Clip domain" connected to the catalytic domain by a 23–92 amino acid linker (Veillard et al., 2016). Their activation relies on a proteolytic cleavage immediately upstream of the catalytic domain. The new N terminus, which contains the consensus sequence I/V-V-G-G-, folds into the enzymatic active site and triggers the catalytic activity (Hedstrom, 2002; Veillard et al., 2016). Because Clip-SPs are organized in cascade, their activation site matches the specificity of the upstream serine protease. Thus, a majority of Clip-SPs present an arginyl or lysyl residue in P1 position upstream of the activation site (Schechter and Berger nomenclature) to be processed by proteases with trypsin-like specificity (Ross et al., 2003; Veillard et al., 2016). Strikingly, Psh differs from most proteases and Clip-SPs in that it contains an unusual histidine in P1 position of the activation site, a residue that can be accepted by the substrate-binding sites of very few proteases. Because the zymogen of Psh (pro-Psh) is sensitive to hydrolysis by the *Beauveria bassiana* protease Pr1A, it has been suggested that pro-Psh could directly be activated by microbial proteases (Gottar et al., 2006). However, the restricted specificity needed to activate Psh is hard to reconcile with the structural and enzymatic diversities of secreted microbial proteases thought to be detected by the danger arm of the Toll pathway. This apparent contradiction prompted us first to determine the nature of the proteases sensed by this arm of the Toll pathway and then to investigate in more detail the molecular mechanism of its activation.

RESULTS

Psh-Dependent Toll Pathway Activation Correlates with the Presence of Microbial Extracellular Proteases

Previous studies have used an array of challenges to monitor the Psh-dependent activation of Toll, ranging from septic injuries with bacteria to natural infections with enthomopathogenic fungi, injection of purified proteases, or ectopic expression of exogenous proteases (El Chamy et al., 2008; Gottar et al., 2006). As a result of this heterogeneity, we do not have a clear picture of the pathogens that activate the Psh pathway. Thus, we used a standardized screen with a unique mode of infection (septic injury by pricking) and a defined microorganism load (OD_{600} from 0.1 to 10) to monitor the activation of either the PRR or the danger arm of the Toll pathway by an array of pathogenic microorganisms. Flies, either wild-type or mutants for Spz (required for both arms), Grass (required for PRR arm only), or Psh (required for danger arm only), were challenged with a panel of infectious microorganisms, and expression of the AMP Drs, a marker of Toll activation, was measured 16 hr post-infection. For the Gram-positive bacteria *Enterococcus faecalis*, *Staphylococcus aureus*, and *Bacillus subtilis* and the fungi *B. bassiana* and *Metarhizium anisopliae*, but also the Gram-negative bacteria *Pseudomonas aeruginosa* and *Serratia marcescens*, we observed a dose-dependent activation of the Toll pathway, which was abolished in spz^{rm7} and psh^{1} null mutants but only weakly affected in $Grass^{hrd}$ null mutant flies (Figure 1). By contrast, the Gram-positive bacteria *Micrococcus luteus*, *Lactobacillus plantarum*, and *Enterococcus faecium* and the fungi *Candida albicans* and *Candida glabrata* did not induce significant *drs* expression under these conditions, although they have been shown to activate Toll through the PAMP pathway when inoculated at high concentrations (Gobert et al., 2003; Gottar et al., 2006; Lemaitre et al., 1997). These results indicate that the Psh pathway can be activated by a range of different microorganisms, including Gram-negative bacteria.

We next used AzoDye collagen, a non-specific protease substrate, to determine the level of extracellular proteolytic activity released by each microorganism in the culture medium. The highest activities were measured in the case of *E. faecalis* and *P. aeruginosa* cultures, while at the opposite no activity could be detected for *Enterobacter cloacae* or *L. plantarum* (Figure 2A). Remarkably, we observed a strict correlation between the levels of secreted proteolytic activity and *drs* expression *in vivo* (Figure 2B). *E. faecalis* strains mutant for the metalloproteinase gelatinase GelE and the serine protease SprE secreted significantly less proteolytic activity, and this correlated with reduced expression of *drs* in infected flies (Figure 2C). Similar results were obtained with a *P. aeruginosa* strain mutant for the elastase LasB (Figure 2D). Altogether, these results reveal that extracellular proteolytic activity is associated with activation of the Toll pathway, independently of the type, structure, specificity, or origin of the secreted proteases.

Processing of pro-Psh by Secreted Microbial Proteases

Because pro-Psh has been shown to be sensitive to hydrolysis by the *B. bassiana* protease Pr1A, we expressed a recombinant form of pro-Psh (rpro-Psh) carrying a C-terminal hexa-histidine Tag in S2 cells. Rpro-Psh was purified from the cell culture supernatant by affinity chromatography, incubated with bacterial and fungal preparations, and analyzed by western blot using an anti-HisTag antibody. As expected, no hydrolysis of rpro-Psh was observed after incubation with microorganisms devoid of extracellular proteolytic activity (Figure 3A). In contrast, incubation with cell-free culture media of pathogens secreting proteases led to rpro-Psh hydrolysis. However, no hydrolysis product could be observed, probably because of the instability of the tag or of the activity of co-secreted carboxypeptidase(s).

A time course analysis on SDS-PAGE gel using Coomassie blue staining revealed a high variability in the early hydrolysis profiles of rpro-Psh incubated in media conditioned by different pathogens. While *E. faecalis*, *S. aureus*, or *B. bassiana* generated stable products, other microorganisms, such as *P. aeruginosa* or *S. marcescens*, seemed to sequentially degrade rpro-Psh (Figure 3B). Identification of the N-terminal extremities of the hydrolysis products by mass spectrometry after in-gel labeling with

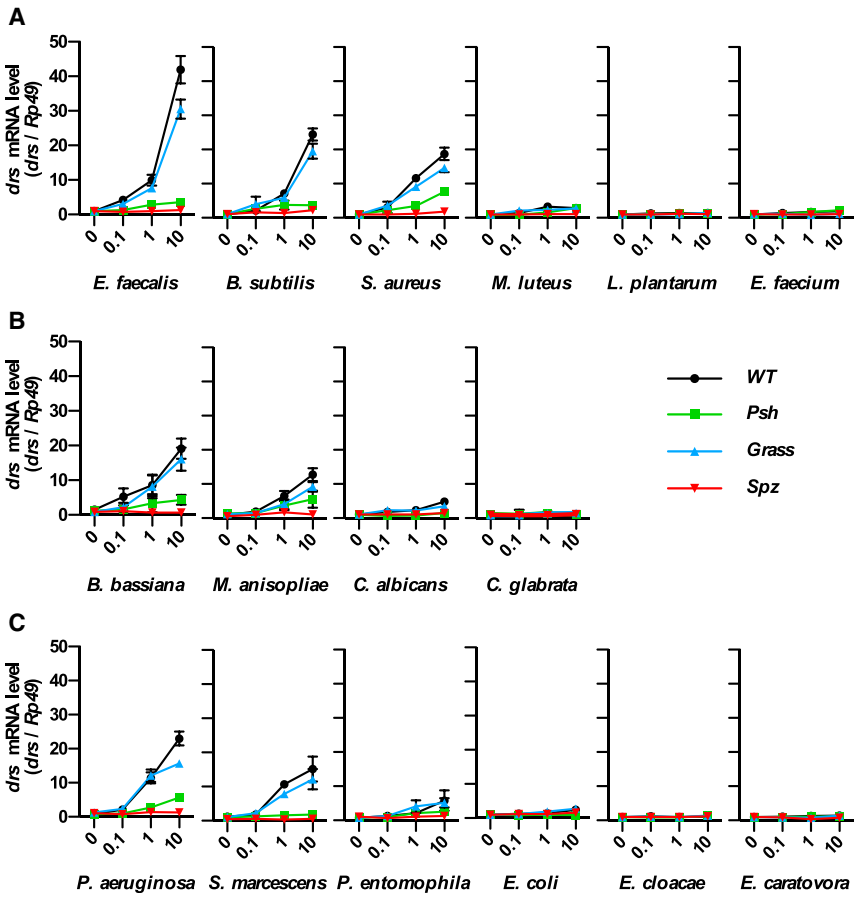


Figure 1. Psh-Dependent Activation of the Toll Pathway

Wild-type w^{1118} flies and $Grass^{hrd}$ (*Grass*), $Spaetzle^{rm7}$ (*Spz*), and psh^{1} (*Psh*) mutant flies were immune challenged by septic injury with three different inocula diluted in PBS (OD_{600} = 0.1, 1, and 10) of Gram-positive bacteria (A), fungi (B), or Gram-negative bacteria (C). PBS (OD_{600} = 0) was used as control. Flies were collected 16 hr after challenge and *drs* gene expression was monitored by qRT-PCR in total RNA extracts. *Ribosomal protein 49* (*Rp49*) mRNA was used as reference gene. Data represent means ± SEs of three independent experiments, each containing three groups of ten flies (five males and five females). Results were normalized to the value obtained for w^{1118} control flies.

TMPP (Ayoub et al., 2015) revealed that only *B. subtilis* was able to release the expected N-terminal extremity of the activated form of the Psh catalytic domain, with a cleavage after the His143 (Figures 3B, 3C, and S1). Furthermore, the Psh catalytic domain released by *B. subtilis* was active when incubated with Z-Arg-AMC, a fluorogenic substrate (Figure 3D). Incubation of rpro-Psh with the *B. subtilis* purified protease subtilisin resulted in cleavage of rpro-Psh at the extremity of the clip domain, generating a proteolytic active form of Psh. Identical results were obtained when the *B. subtilis* subtilisin was incubated with a catalytically inactive mutant version of pro-Psh containing a substitution of Ser338 in the protease active site (Figure S2). This confirms that release of the Psh catalytic domain by subtilisin is direct and does not implicate an auto-processing event.

By contrast, no proteolytic activity could be detected for rpro-Psh incubated with the media conditioned by the other pathogens, confirming the unique capacity of the *B. subtilis* to effectively and directly activate Psh (Figure 3D). Strikingly, however, the first hydrolysis products generated during incubation with all other microbial conditioned media always corresponded to cleavage(s) in the same region (G_{107}-G_{118}) of the Clip domain (Figure 3B). This indicates that this short sequence is highly susceptible to hydrolysis, independently of the specificity of the attacking protease. This region may thus act as bait for microbial proteases.

Cleavage in a Bait Region of the Clip Domain Is Essential for the Activation of Psh by Microbial Proteases

We hypothesized that cleavage in the "bait region" of the Clip domain may constitute the first step of a Psh sequential activation. To test this hypothesis, we constructed pro-Psh mutants in which this hypothetic bait region was substituted partially (rpro-Psh^{M1}) or totally (rpro-Psh^{M2}) by alanyl residues (Figure 4A). *In vitro*, these mutations significantly reduced the cleavages in the bait region by both *E. faecalis* conditioned medium and *B. subtilis* subtilisin. However, they did not affect the release of the specific active Psh catalytic domain upon incubation with *B. subtilis* subtilisin (Figure 4B). Interestingly, *E. faecalis* proteases reported their activity within the catalytic domain, with a cleavage after the Gly202. In parallel, wild-type rpro-Psh, rpro-Psh^{M1}, and rpro-Psh^{M2} were expressed in psh^{1} null mutant flies under the control of a fat body-specific promoter (Figure 4C). Expression of wild-type rpro-Psh restored Toll pathway activation upon *B. subtilis* subtilisin injection or *E. faecalis* infection (Figures 4D and 4E). Expression of rpro-Psh^{M1} and rpro-Psh^{M2} also allowed almost full restoration of the Toll pathway inducibility to the *B. subtilis* subtilisin in the psh^{1} mutant background. However, neither of the mutant forms rescued *drs* expression after *E. faecalis* infection (Figure 4D). These results demonstrate the essential function of the bait region for Psh activation by extracellular microbial proteases and confirm the unique mode of action of *B. subtilis* subtilisin.

A Cysteine Protease Inhibitor Blocks Psh-Dependent, but Not Grass-Dependent, Toll Pathway Activation

The results above suggest a sequential activation of pro-Psh involving an endogenous protease that would cleave the remaining amino acids of the Psh pro-domain at His143. Among the proteases secreted into the *Drosophila* hemolymph, serine proteases do not possess the specificity needed to accept a histidine in P1 position, and various approaches performed in the

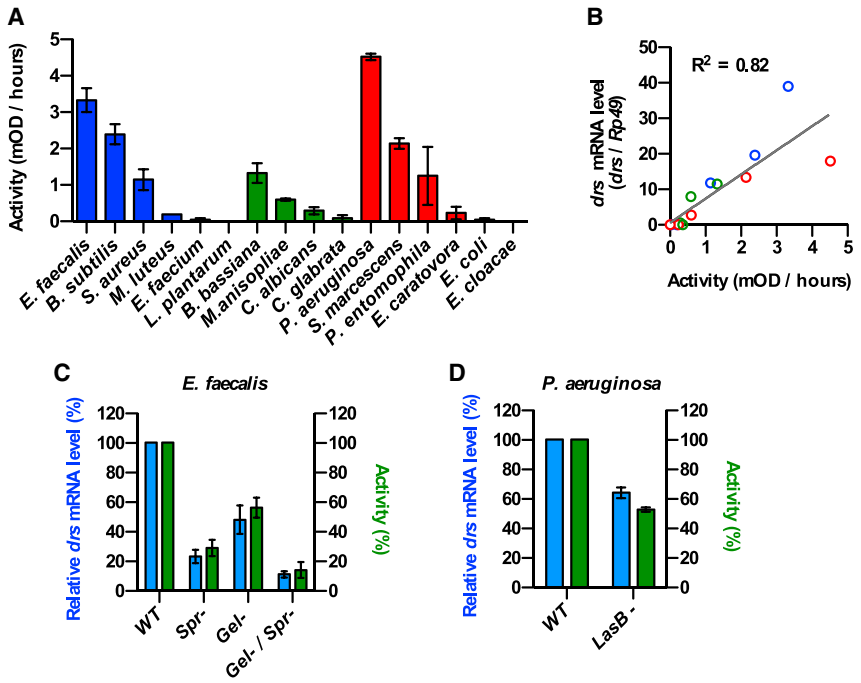


Figure 2. Psh-Dependent Toll Pathway Activation versus Microbial Proteolytic Activity

(A) Extracellular proteolytic activity of Gram-positive bacteria (blue), fungi (green), and Gram-negative bacteria (red) grown to early stationary phase was determined on the non-specific substrate AzoDye collagen (15 mg/mL) in 0.1 M TrisHCl buffer (pH 8). Data represent means ± SEs of three independent experiments.

(B) Psh-dependent induction of *drs* expression upon immune challenge (OD_{600} = 10) from Figure 1 was correlated to the respective proteolytic activity determined on AzoDye collagen.

(C and D) Extracellular proteolytic activity and Psh-dependent induction of *drs* expression were determined under the same conditions as above for *E. faecalis* serine protease E (SprE) and gelatinase (GelE) null mutant strains (C) or for the *P. aeruginosa* elastase (LasB) null mutant (D) and expressed relative to the values obtained from the respective parental strains.

Data represent means ± SEs of three independent experiments.

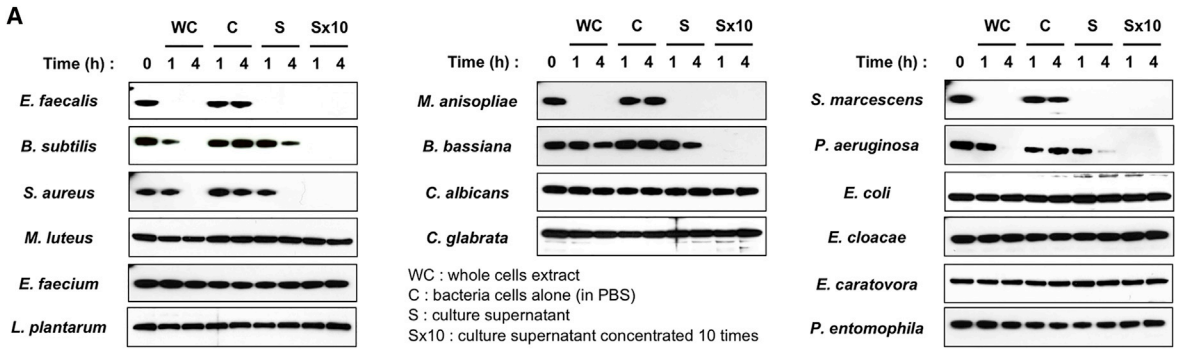
laboratory did not reveal immune function for the two matrix-metalloproteases in Toll pathway activation. Extracellular cysteine proteases, particularly cathepsins, represent an interesting alternative and have been suggested in the past to participate in insect immunity (Fujimoto et al., 1999; Serbielle et al., 2009).

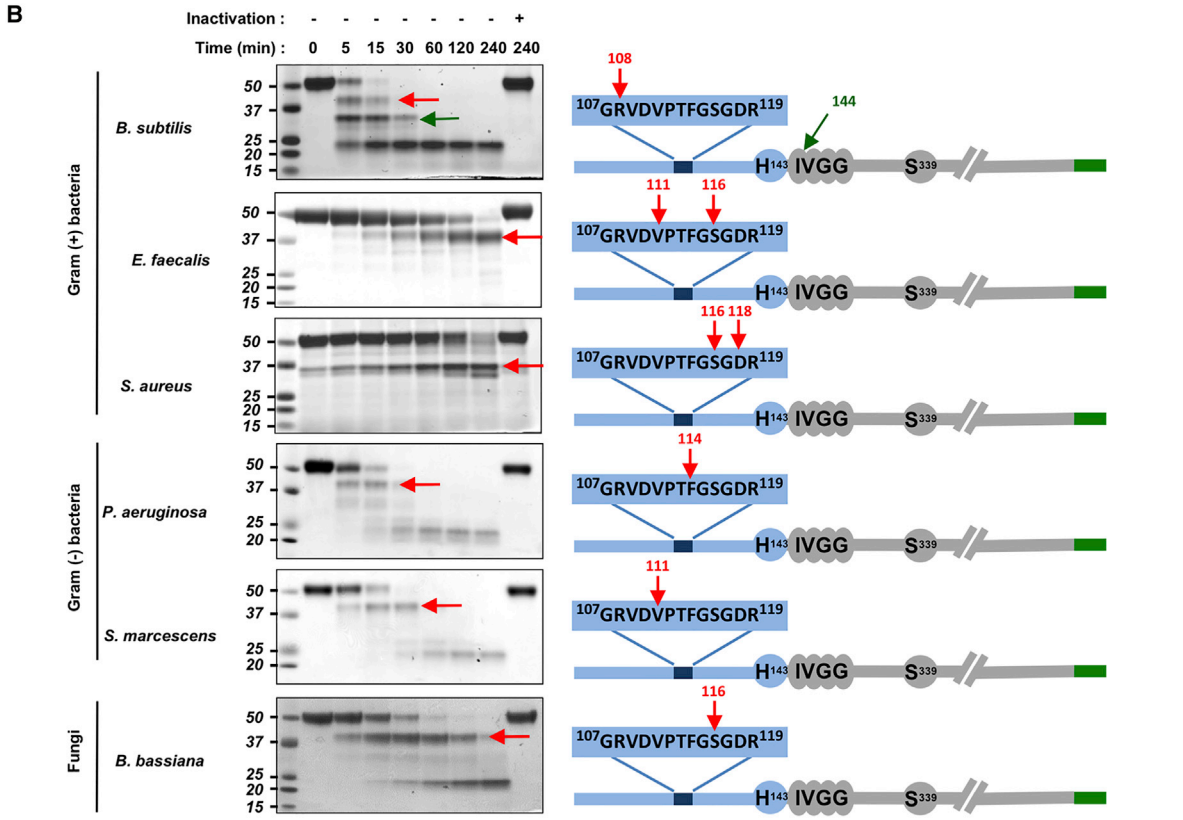
In order to evaluate their implication in Toll pathway activation, we injected the cysteine protease inhibitor E-64 into the body cavity of wild-type flies 2 hr before immune challenge and monitored *drs* expression (Figure 5). E-64 blocked Toll pathway activation in response to *E. faecalis* or *B. bassiana* as efficiently as Psh inactivation (Figures 5A and 5B). However, it only had a limited impact following *M. luteus* infection (Figure 5C). E-64 totally suppressed the Psh-dependent Toll pathway activation following microbial challenge when injected in *Grass*^{hrd} mutant flies, but had no additional impact when injected into Psh null mutant flies. Moreover, E-64 totally blocked activation of the Toll pathway in response to purified *A. oryzae* protease in wild-type flies, but did not affect the response to injection of purified *M. luteus* PGNs (Figures 5D and 5E). Importantly, E-64 injection had only a marginal impact on the activation of Toll pathway upon injection of *B. subtilis* subtilisin (Figure 5F). Altogether, these data reveal that a cysteine protease inhibited by E-64 participates in the activation of pro-Psh by microbial proteases unable to directly cleave at His143 as *B. subtilis* subtilisin does.

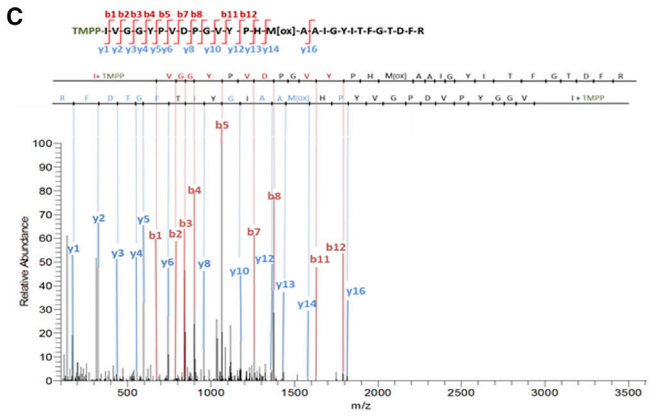
Identification and Characterization of 26-29-p, a Cathepsin Involved in Psh-Dependent Toll Pathway Activation

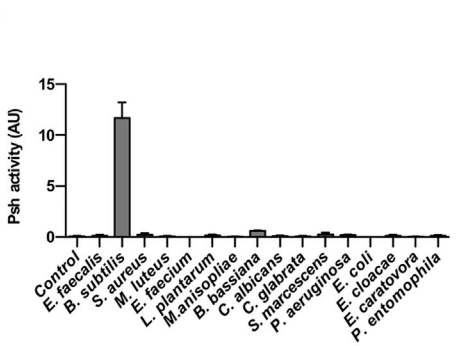
We next monitored activation of the Toll pathway in fly lines in which the seven *Drosophila* cathepsins or the bleomycin hydrolase, all known to be sensitive to E-64, were inactivated by transposon insertion or RNA interference. The flies harboring a P-element inserted in the gene encoding the 26-29-protease (26-29-p: CG8947) showed reduced *drs* expression following infection with *E. faecalis*, *M. luteus*, or *B. bassiana* (Figures S3A–S3C). This reduction was comparable to that observed in *psh*^{1} mutant flies. CG8947 mutant flies also displayed an increased susceptibility to infections by *E. faecalis* and *B. bassiana* (Figures S3D and S3E). Remarkably, while most cysteine cathepsins are intracellular and present a short and unstructured inhibitory pro-fragment, cathepsin 26-29-p contains both a signal peptide and a 26 kDa N-terminal pro-domain of unknown function and has been found in the circulating system of various insects (Fujimoto et al., 1999; Serbielle et al., 2009).

To characterize the function of the cathepsin 26-29-p, we generated additional mutant alleles by imprecise excision of the P-element inserted in the first exon of the *26-29-p* gene. We obtained two new alleles, *26-29-p*^{H3} and *26-29-p*^{H6}, carrying deletions of the *26-29-p* coding region as well as a revertant *26-29-p*^{A2} resulting from a clean excision of the P-element (Figure S4). Toll pathway activation was fully restored in the *26-29-p*^{A2} flies, confirming that the phenotype we observed was caused by the insertion in the *26-29-p* gene. *26-29-p*^{H3} and *26-29-p*^{H6} null mutant flies showed reduced activation of the Toll pathway 24 hr after immune challenge with *E. faecalis* or *B. bassiana*, similar to *psh*^{1} mutants (Figures 6A and 6B). This reduction was not observed upon infection with *M. luteus* bacteria or following injection of purified *M. luteus* PGNs (Figures 6C and 6D). Moreover, activation of the Toll pathway was totally abolished in *26-29-p*^{H3} and *26-29-p*^{H6} flies in response to infection with Gram-negative *P. aeruginosa* bacteria or injection of purified protease from *A. oryzae* (Figures 6E and 6F). By contrast,









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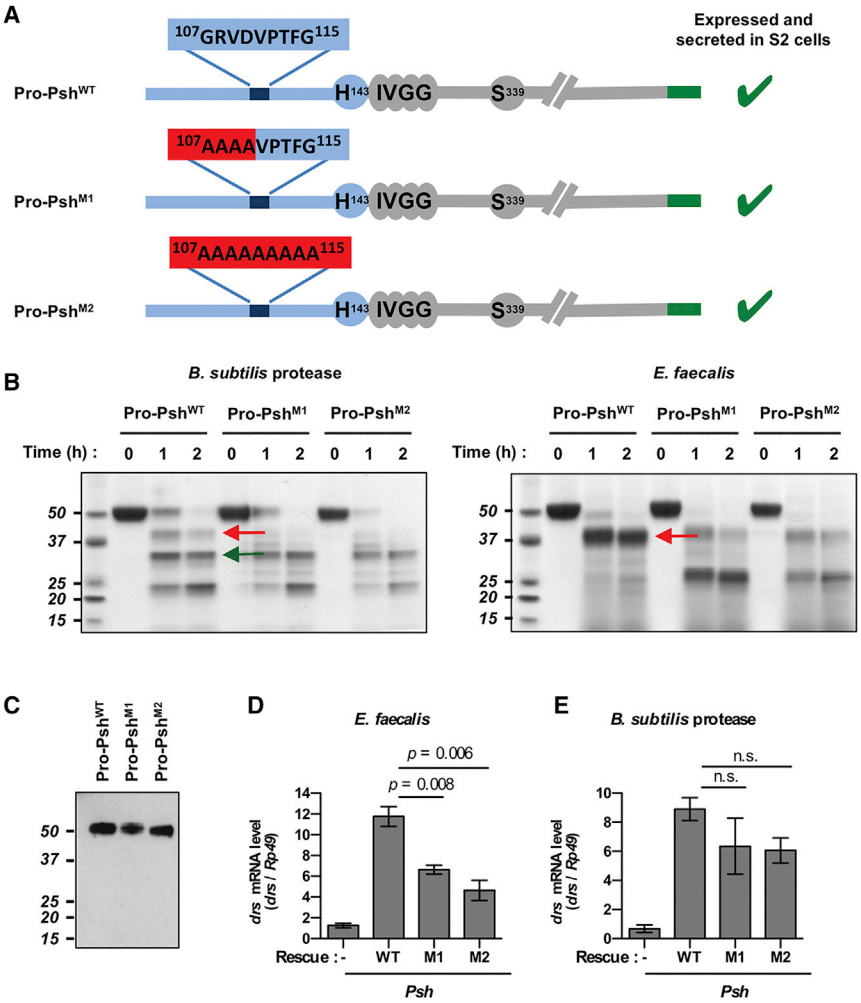

Figure 4. Psh Inactivation by Mutations in the Bait Region

(A) Structure of pro-Psh mutants with partial (pro-Psh^{M1}) or total substitution (pro-Psh^{M2}) of the bait region by alanyl residues.

(B) Purified mutant or wild-type rpro-Psh (0.2 μg/μL) was incubated for 1 or 2 hr at 29°C with cell-free *E. faecalis* culture medium supernatant or with purified *B. subtilis* protease (1 nM). Following electrophoresis, hydrolysis products were visualized by Coomassie blue staining. Red arrows indicate fragments resulting from hydrolysis in the bait region and the green arrow indicates the active catalytic domain.

(C–D) Wild-type pro-Psh, pro-Psh^{M1}, and pro-Psh^{M2} were expressed under the control of the fat body *Yolk* driver in *psh*^{1} mutant flies.

(C) Secretion of wild-type and mutant pro-Psh in the blood was determined by western blot using an anti-6HisTag antibody.

(D and E) *drs* mRNA levels were monitored by qPCR in *psh*^{1} mutant flies (*psh*^{1}*; yolk-GAL4/+*) expressing the wild-type rpo-Psh (WT; *psh*^{1}; *yolk-GAL4/UAS-pro-Psh*), the M1 mutant (M1; *psh*^{1}; *yolk-GAL4/UAS-pro-Psh*^{M1}), and the M2 mutant (M2; *psh*^{1}*; yolk-GAL4/UAS-proPsh*^{M2}) for 24 hr at 25°C following *E. faecalis* challenge (OD_{600} = 1) (D) or 16 hr at 25°C following *B. subtilis* protease injection (E).

Data represent means ± SEs of three independent experiments, each containing three groups of ten flies (five males and five females). p values obtained from Student's t test are indicated on the graphs.

deletions in the *26-29-p* gene only partially reduced Toll pathway activation upon injection of the purified subtilisin from *B. subtilis* (Figure 6G). Finally, *26-29-p*^{H3} and *26-29-p*^{H6} mutants, but not *26-29-p*^{A2} revertant flies, showed a similar increased susceptibility to infection by *E. faecalis* and *B. bassiana* as *psh*^{1} mutant flies (Figure 6H). We conclude that the 26-29-p cathepsin is required for activation of Psh by microbial proteases, with the exception of those that can directly activate it, such as *B. subtilis* subtilisin.

Sequential Activation of Psh by Microbial Proteases and Cathepsin 26-29-p

To confirm these genetic results, we expressed a recombinant form of the pro-cathepsin 26-29-p carrying a C-terminal

Figure 3. Hydrolysis of rpro-Psh by Microbial Extracellular Proteases

(A) rpro-Psh labeled with a C-terminal HisTag (0.2 μg/μL) was incubated at 29°C with whole-cell cultures of microorganisms grown to early stationary phase and microorganism cells suspended only in PBS, cell-free culture medium (supernatant), or the same medium (supernatant) concentrated ten times. After 1 or 4 hr, 1 μg aliquots were removed and the reaction was stopped at 100°C for 5 min. Residual rpro-Psh was visualized by western blot with an anti-6HisTag antibody. Representative results of at least two independent experiments.

(B) rpro-Psh was incubated under the same conditions with cell-free media (supernatant). At various time points, 5 μg of proteins were removed and the reaction was stopped at 100°C for 5 min. Controls were performed after pre-inactivation of the media for 5 min at 100°C. Hydrolysis products were visualized by Coomassie blue staining after SDS-PAGE electrophoresis. Identifications of neo-N-terminal peptides were determined by nano-liquid chromatography-tandem mass spectrometry (nano-LC-MS/MS) analysis after in-gel protein N-terminal chemical derivatization method using TMPP reagent. Arrows indicate identified N-terminal extremities.

(C) Characterization of TMPP-derivatized peptide of the expected N-terminal extremity of the catalytic domain by MS/MS fragmentation spectrum. The corresponding MS/MS fragmentation table is presented as Table S4.

(D) Cell-free supernatant of S2 cells expressing rpro-Psh (200 μL) was incubated in 0.1 M TrisHCl buffer (pH 8) with 200 μL of cell-free medium from microorganism cultures (final volume, 600 μL). After 1 hr, proteolytic activity of the generated rpro-Psh hydrolysis products was determined on the fluorogenic substrate Z-Arg-AMC for 30 min at 29°C in 0.1 M TrisHCl buffer (pH 8) supplemented with 5 mM $CaCl_2$.

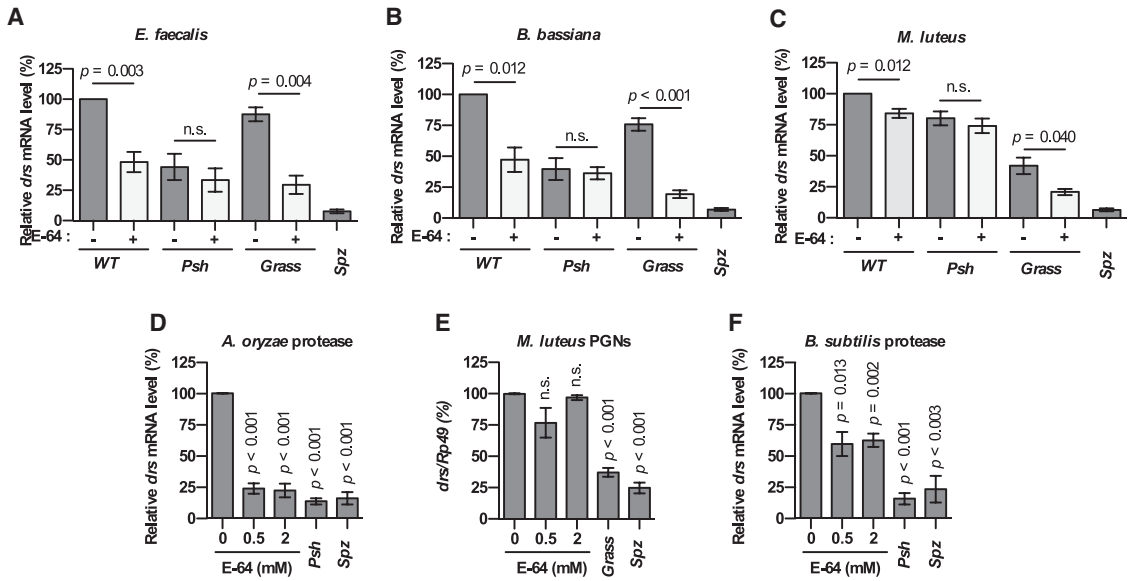

Figure 5. The Cysteine Protease Inhibitor E-64 Blocks Psh-Dependent Induction of *drs* Expression upon Immune Challenge

(A–C) A total of 18.4 nL of PBS alone or containing 2 mM of the cysteine protease inhibitor E-64 were injected into w^{1118} (*WT*), psh^{1} (*Psh*), and $Grass^{hrd}$ flies (*Grass*). spz^{rm7} mutant flies (*Spz*) were used as control. After 2 hr, flies were immune challenged for 24 hr by septic injury with *E. faecalis* (OD_{600} = 1) (A), by natural infection with *B. bassiana* (B), or by septic injury with *M. luteus* (OD_{600} > 200) (C).

(D–F) The E-64 inhibitor was injected at 0.5 or 2 mM into w^{1118} flies and $Grass^{hrd}$, psh^{1}, and spz^{rm7} mutant flies were used as control. After 2 hr, flies were challenged for 16 hr with *A. oryzae* protease (D), for 24 hr with *M. luteus* peptidoglycans (E), and for 16 hr with *B. subtilis* protease (F). Flies were collected and *drs* gene expression was monitored by qRT-PCR in total RNA extracts. *Ribosomal protein 49* (*Rp49*) mRNA was used as reference gene. Results were normalized to the value obtained with the control conditions.

Data represent means ± SEs of three independent experiments, each containing three groups of ten flies (five males and five females). p values obtained from Student's t test are indicated on the graphs.

hexa-histidine Tag in *Drosophila* S2 cells. In agreement with its proposed extracellular function, rpro-cathepsin 26-29-p was not detected in the cell extract but was exclusively found in the culture media (Figure S5A). To activate rpro-cathepsin 26-29-p, we incubated the concentrated supernatant with pepsine at an acidic pH following the common procedure for recombinant pro-cathepsin activation (Brömme et al., 2004). Under these conditions, rpro-cathepsin 26-29-p was processed and the proteolytic activity of its generated active form could be followed on the fluorogenic substrate Z-FR-AMC (Figures S5B and S5C).

We then incubated the activated cathepsin 26-29-p extract with the full-length rpro-Psh or after its pre-processing into the bait region by *E. faecalis* culture media (Figures 7A and 7B). Remarkably, under our conditions, cathepsin 26-29-p had no effects on the full-length pro-Psh but cleaved the pre-processed form following a hydrolysis pattern similar to the one already observed after incubation with *B. subtilis*. Indeed, mass spectrometry analysis confirmed that cathepsin 26-29-p released the active form of Psh with a first cleavage at the extremity of the Clip domain after the His143. In agreement with these results, cathepsin 26-29-p was not able to cleave the partially processed rpro-Psh mutant containing a substitution of His143 in the same conditions (Figure 7C).

Altogether with the genetic data, these results confirmed the function of cathepsin 26-29-p in the sequential activation of Psh during infections.

DISCUSSION

A Pathway for Sensing an Array of Microbial Proteolytic Activities

Two theories were proposed in the late 1980s and early 1990s to account for activation of innate immunity by a limited number of receptors. Heralded by C. Janeway and P. Matzinger, these theories postulated that innate immunity was activated by non-self and danger signals, respectively (Janeway, 1989; Matzinger, 1994). During infections, damage to host tissues and cells leads to the release of intracellular molecules that can activate innate immunity upon binding PRRs (e.g., activation of TLR3, 7, 8, and 9 by self DNA or RNA or of the C-type lectin receptor DNGR1 by F-actin) (Ahrens et al., 2012; Zelenay and Reis e Sousa, 2013). Conserved from flies to mammals, damage-induced responses are important for triggering tissue repair, but they are delayed and occur when pathogen progression already impacts the host integrity (Allen and Wynn, 2011; Iwasaki and Medzhitov, 2015; Srinivasan et al., 2016). Of note, danger can be sensed before damage occurs, through monitoring the activity of microbial molecules associated with pathogenesis. Sometimes referred to as effector-triggered immunity, this strategy of surveillance focuses on toxins or virulence factors. For example, pore-forming toxins activate NLRP3 and the inflammasome in mammals, while the cytotoxic necrotizing factor-1 from *E. coli* modifies the enzyme Rac2, triggering its interaction with IMD and induction of AMPs (Boyer et al., 2011; Martinon et al.,

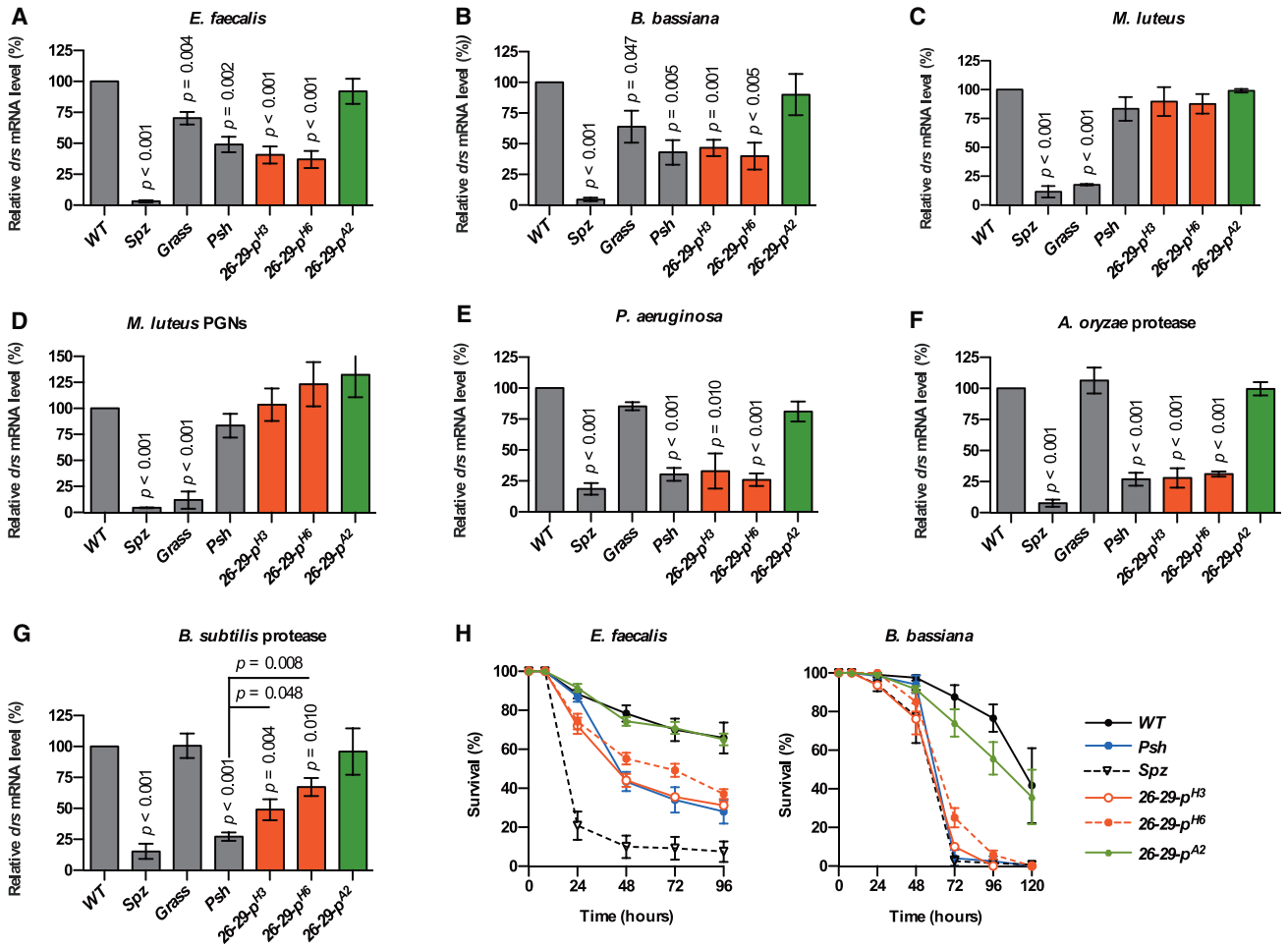

Figure 6. Cathepsin 26-29-p Is Required for Psh Sequential Activation

Two null mutant alleles of the *26-29-p* gene (*26-29-p^{H3}* and *26-29-p^{H6}*) were generated by imprecise P-element excision resulting in deletions spanning transcriptional start site and the first two exons of the gene. A clean excision restoring expression of wild-type *26-29-p* was used as control (*26-29-pA2*) (Figure S4).

(A–G) Flies of the indicated genotype were challenged by septic injury with *E. faecalis* (OD_{600} = 1) (A), *M. luteus* (OD_{600} > 200) (C), or *P. aeruginosa* (OD_{600} = 1) (E); by natural infection with *B. bassiana* (B); or by injection of *M. luteus* peptidoglycans (D), *A. oryzae* protease (F), or *B. subtilis* protease (G). After 24 hr at 29°C (16 hr for the purified proteases), flies were collected and *drs* gene expression was monitored by qRT-PCR in total RNA extracts. *Ribosomal protein 49* (*Rp49*) mRNA was used as reference gene. Results were normalized to the value obtained with the wild-type flies. Data represent means ± SEs of three independent experiments, each containing three groups of ten flies (five males and five females). p values obtained from one-way ANOVA test are indicated on the graphs.

(H) Survival of adult flies challenged with *E. faecalis* by septic injury (OD_{600} = 1) or with *B. bassiana* by natural infection. Data represent means ± SEs of three independent experiments, each containing three groups of 20 flies (10 males and 10 females). Log-rank statistical analyses are presented as Table S5.

2009). Defense reactions can also be activated in numerous hosts upon sensing microbial proteases (e.g., Chavarría-Smith et al., 2016; Cheng et al., 2015; de Zoete et al., 2011; Turk, 2007). Notably, in human type 2 immune response is activated by allergens such as the dust mite cysteine cathepsin or by excreted proteases from multicellular parasites by a yet unknown mechanism (Medzhitov and Janeway, 2002).

One critical aspect of innate immunity is to reconcile sensing of an immense range of potential microbial inducers with a restricted number of receptors (Janeway, 1989). We now have a reasonable understanding of the way this is achieved for PRRs. However, up to now it has remained unclear how this occurs in the context of virulence factors, which often target specific host molecules. This is particularly true for pathogen proteases due to their high enzymatic specificities. The identification of a critical region in the pro-domain of Psh, functioning as bait for microbial proteases independently of their origin, type, or specificity, provides for the first time an example of an innate immunity receptor able to sense a broad range of microbes through virulence factors, rather than molecular patterns.

Under physiological conditions, our previous studies have shown that the serine protease inhibitors of the serpin family such as Necrotic (Nec) control the residual activation of the danger arm by endogenous proteases since *Nec* null mutant flies display a constitutive and Psh-dependent activation of the Toll pathway (Levashina et al., 1999; Ligoxygakis et al., 2002).

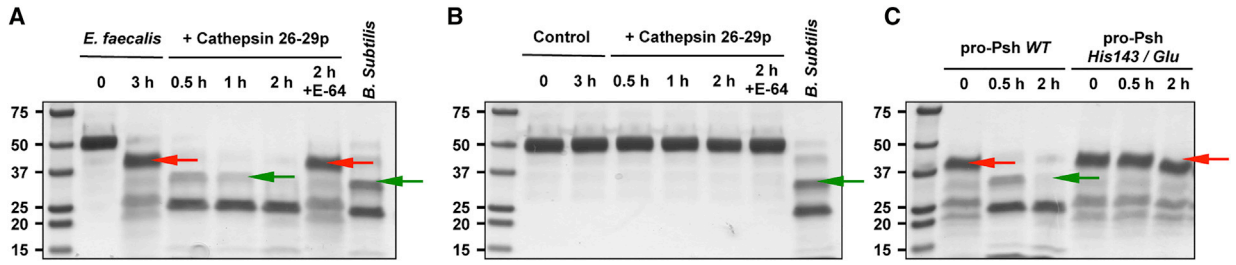

Figure 7. Sequential Activation of rpro-Psh by Bacterial Proteases and Cathepsin 26-29-p

(A and B) Purified rpro-Psh was incubated in 0.1 M Tris buffer (pH 8) with (A) or without (B) *E. faecalis* culture supernatant at 29°C. After 3 hr, the partially processed rpro-Psh was incubated with the pre-activated cathepsin 26-29-p for 0.5–2 hr at 29°C in 0.2 M sodium acetate buffer (pH 5.5). The generated Psh hydrolysis products were then visualized by Coomassie blue staining after SDS-PAGE electrophoresis. Incubation under the same conditions with cathepsin 26-29-p pre-inactivated with E-64 was used as control. For comparison, hydrolysis products generated by *B. subtilis* subtilisine generated as previously described are presented. The N-terminal extremities of the hydrolysis products of interest were determined by N-terminal labeling and mass spectrometry. Red arrows indicate N-terminal extremities localized in the bait region, and the green arrows show the expected N-terminal extremity of the active form of Psh.
(C) Alternatively, the experiment was repeated with rpro-Psh His143/Glu.

However, while we focused our study on exogenous proteases, we can hypothesize that the lack of specificity of the Psh activation mechanism allows the sensing of abnormal concentration of endogenous proteases in the hemolymph. Indeed, a Psh-dependent activation of the Toll pathway was observed after overexpression of an active form of the Grass serine protease in the hemolymph as well as after necrosis triggering (El Chamy et al., 2008; Ming et al., 2014).

A Unique Mode of Activation among Serine Proteases

The mechanism solved here involves sequential activation of Psh, with an initial cleavage in the bait region by microbial proteases licensing the subsequent maturation of Psh to the endogenous cathepsin 26-29-p. Since peptide bond hydrolysis is irreversible, proteolytic enzymes are tightly regulated at the transcriptional and post-translational levels (Khan and James, 1998). Two-step processes are frequently observed for activation of zymogens belonging to the chymotrypsin family as they allow strict spatial (i.e., activation of neutrophil and mast cell serine proteases in the azurophilic granules) or temporal regulation (i.e., activation of plasmin and thrombin during fibrinolysis and coagulation, respectively) (Caughey, 2016; Collen, 1999; Korkmaz et al., 2008; Wood et al., 2011). However, in most cases, sequential activation involves two highly specific cleavage sites not compatible with the sensing of a broad range of proteases. Hence, the long bait region highly sensitive to proteolysis described here constitutes an original strategy to detect exogenous proteases independent of their specificities.

Interestingly, the mode of activation of Psh is reminiscent of the mechanism of inhibition by α_2-macroglobulin (α_2-M), a non-specific inhibitor targeting both self- and non-self-proteases and clearing them from the tissue fluids (Garcia-Ferrer et al., 2017; Goulas et al., 2017). Indeed, α_2-M contains a 25 amino acid-long bait region, which is also sensitive to all classes of proteases. Upon cleavage of this bait region, α_2-M undergoes a structural rearrangement, thus trapping the target protease(s) (Marrero et al., 2012).

Overall, the proposed model is evocative of the guard system in plants, where structural modification of host proteins by pathogen effectors (here, Psh) is sensed by guard receptors (here, the cathepsin 26-29-p) to trigger an appropriate immune response (Jones and Dangl, 2006).

An Essential Immune Function for a Circulating Cathepsin

Cysteine cathepsins have long been known to participate in intracellular protein turnover inside the endosome/lysosome compartments. However, it has now been shown that in specific physiological or pathological conditions they can be addressed to alternative intracellular localizations or even extracellular space (Brix et al., 2008). Remarkably, we visualized the recombinant cathepsin 26-29-p only in the cell supernatant, but not in the cellular lysate. This observation corroborates previous studies that described the presence of cysteine 26-29-p homologs in the hemolymph of the Lepidopteran *Manduca sexta* and the flesh flies *Sarcophaga peregrina* (Fujimoto et al., 1999; Serbielle et al., 2009). This specific extracellular localization could be due to the 26 kDa N-terminal pro-domain of unknown function that is unique among cysteine cathepsins.

In mammals, cysteine cathepsins are involved in both adaptive and innate immunity. In addition to their main role in the degradation of phagocytized microbes in the phago-lysosomes, cathepsins are also essential for the regulation of MHC class II-dependent antigen presentation (Sadegh-Nasseri and Kim, 2015). In the innate immune system, cathepsins are mandatory for addressing TLR7 and TLR9 to the reticulum and for the post-translational processing of several cytokines (e.g., IL-8 or TNF-alpha) (Ewald et al., 2008; Ha et al., 2008; Ohashi et al., 2003; Park et al., 2008). Of note, cathepsin C and cathepsin L participate in the activation of serine proteases in the context of immune responses (i.e., neutrophil serine proteases and granzymes for cathepsin C and complement protease C3 for cathepsine L) (Hamon et al., 2016; Liszewski et al., 2013).

In addition, cysteine cathepsins are found extracellularly in inflammatory conditions, although the biological significance of this observation is still unclear. Associated with deleterious effects, such as degradation of the extracellular matrix or basal

membrane components leading to the loss of tissue integrity, extracellular cathepsins may represent a bystander event rather than a specific response to infection and have even been considered as potential therapeutic targets in chronic inflammatory diseases (Vasiljeva et al., 2007). Our results described for the first time the implication of a circulating cysteine cathepsin in the model organism *Drosophila melanogaster* innate immune system. They suggest that the immune function of extracellular cysteine cathepsins in mammals is a topic that deserves further attention.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and five tables and can be found with this article online at https://doi.org/10.1016/j.molcel.2018.01.029.

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AUTHOR CONTRIBUTIONS

F.V., J.-M.R., and N.M. designed the study, interpreted the results, and wrote the paper. N.I., E.L., and F.V. performed most of the experiments. N.G., C.S.-R., and A.V.D. designed, performed, and interpreted the mass spectrometry analysis.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-His (C-term) Antibody	Invitrogen	#P/N 46-0693
Bacterial and Virus Strains		
Escherichia coli ATCC23724	ATCC	ATCC23724
Enterobacter cloacae	H. Monteil lab (University Louis Pasteur, Strasbourg)	N/A
Staphylococcus aureus RN6390	H. Monteil lab (University Louis Pasteur, Strasbourg)	N/A
Serratia marcescens Db11 20C2	Nehme et al., 2007	20C2
Bacillus subtilis	J. Millet lab (Pasteur institute of Paris)	N/A
Erwinia carotovora Ecc15	Basset et al., 2000	N/A
Enterococcus faecalis OG1RF	E. Murray lab (University of Texas) Kawalec et al., 2005	TX4002
E. faecalis gelE-	E. Murray lab (University of Texas) Kawalec et al., 2005	TX5264
E. faecalis sprE-	E. Murray lab (University of Texas) Kawalec et al., 2005	TX5243
E. faecalis gelE- and *sprE-*	E. Murray lab (University of Texas) Kawalec et al., 2005	TX5128
Enterococcus faecium	E. Murray lab (University of Texas) Kawalec et al., 2005	N/A
Pseudomonas aeruginosa PA14	F.M. Ausubel lab; Liberati et al., 2006	N/A
Pseudomonas aeruginosa; Elastase null mutant	F.M. Ausubel lab; Liberati et al., 2006	*ID31939*
Pseudomonas aeruginosa; Protease IV null mutant	F.M. Ausubel lab; Liberati et al., 2006	*ID37740*
Pseudomonas entomophila	B. Lemaitre lab (University of Lausanne)	N/A
Micrococcus luteus ATCC4698	ATCC	ATCC4698
Lactobacillus plantarum	W.J. Lee lab (Seoul National University)	N/A
Beauveria Bassiana 80.2	Gottar et al., 2006	N/A
Metarhizium anisopliae V275	Gottar et al., 2006	N/A
Candida glabrata	Gottar et al., 2006	N/A
Candida albicans	Isolated by M. Koenig (CHU Strasbourg-Hautepierre)	N/A
Chemicals, Peptides, and Recombinant Proteins		
rpro-Psh^{WT}	This paper	N/A
rpro-PshM1	This paper	N/A
rpro-PshM2	This paper	N/A
rpro-Psh Ser339/Ala	This paper	N/A
rpro-Psh His144/Glu	This paper	N/A
rpro-Psh His144/Glu: Ser339/Ala	This paper	N/A
Protease, from Bacillus Sp.	Sigma	#P5985
Protease, from Aspergillus oryzae	Sigma	#P6110
Peptidoglycan from Micrococcus luteus	Sigma	#53243
Pepsine from porcine gastric mucosa	Sigma	#P6887
Azo dye-impregnated collagen	Sigma	#A4341
Z-Arg-MCA	Sigma-aldrich	#C8022
Z-Phe-Arg-AMC	Bachem	I-1160
(N-succinimidyloxycarbonyl-methyl)tris-(2,4,6-trimethoxyphenyl)phosphonium bromide (TMPP)	Ayoub et al., 2015	N/A
Porcine trypsin	Promega	#V5111
Deposited Data		
Images of gels and blots	Mendeley data	https://doi.org/10.17632/mzgnrcftzv.1

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental Models: Cell Lines		
Drosophila S2 cells	Invitrogen	#R69007
Experimental Models: Organisms/Strains		
D. melanogaster w^{1118}	El Chamy et al., 2008	N/A
D. melanogaster psh^{1}	El Chamy et al., 2008	N/A
D. melanogaster $grass^{hrd}$	El Chamy et al., 2008	N/A
D. melanogaster spz^{rm7}	El Chamy et al., 2008	N/A
D. melanogaster C564-gal4 driver	Bloomington stock center	#6982
D. melanogaster 26-29-$p^{KG00154}$	Bloomington stock center	#13051
D. melanogaster 26-29-pH3	This paper	N/A
D. melanogaster 26-29-pH6	This paper	N/A
D. melanogaster 26-29-pA2	This paper	N/A
D. melanogaster UAS-pro-Psh^{WT}	This paper	N/A
D. melanogaster UAS-pro-Psh^{M1}	This paper	N/A
D. melanogaster UAS-pro-Psh^{M2}	This paper	N/A
D. melanogaster ATT-3B VK00033	Bloomington stock center	#24871
P(SUPor-P)26-29-$p^{KG00154}$	Bloomington stock center	#13051
P(TRIP.HMS00725)attP2	Bloomington stock center	#32932
P(TRIP.HMS02491)attP2	Bloomington stock center	#42655
P(TRIP.HMS00910)attP2	Bloomington stock center	#33955
Mi(MIC)CG11459^{MI08810}	Bloomington stock center	#50488
P(EPgy2)CtsB1^{EY03339}	Bloomington stock center	#15434
P(TRIP.GL00551)attP2	Bloomington stock center	#36591
P(SUPor-P)CG1440^{KG04580}	Bloomington stock center	#13977
Recombinant DNA		
pMT/V5-His vector	Invitrogen	#V412020
pJM1345: rpro-Psh^{WT} in pMT/V5-His	this paper	N/A
pJM1681: rpro-Psh^{M1} in pMT/V5-His	this paper	N/A
pJM1682: rpro-Psh^{M2} in pMT/V5-His	this paper	N/A
pUAST-attB vector	Bischof et al., 2007	N/A
pJM1692: pro-Psh^{WT} in pUAST-attB	this paper	N/A
pJM1693: pro-Psh^{M1} in pUAST-attB	this paper	N/A
pJM1694: pro-Psh^{M2} in pUAST-attB	this paper	N/A
pJM1674: rpro-Psh Ser339/Ala in pMT/V5-His	this paper	N/A
pJM1675: rpro-Psh His144/Glu in pMT/V5-His	this paper	N/A
pJM1676: rpro-Psh His144/Glu: Ser339/Ala in pMT/V5-His	this paper	N/A
pJM1689: rpro-cathepsin 26-29-p in pMT/V5-His	this paper	N/A
Software and Algorithms		
GraphPad Prism 05	GraphPad Software	N/A
Mascot algorithm v2.5.1	Matrix Science	N/A
Scaffold software	Proteome Software	N/A
OASIS online application	Yang et al., 2011	N/A

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Florian Veillard (f.veillard@ibmc-cnrs.unistra.fr).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Fly strains

All *Drosophila melanogaster* were reared at 25°C on standard cornmeal-agar medium in a 12-hour light-dark cycle. w^{1118} flies were used as wild-type control throughout the experiments. psh^{1}, $Grass^{hrd}$ and spz^{rm7} mutant flies have been described previously (El Chamy et al., 2008). The *C564-gal4* driver (#6982) is from Bloomington stock center. Cathepsin mutant flies are described in Table S3. The 26-29-p^{H3}, 26-29-p^{H6} and the 26-29-p^{A2} mutants were created by excision of the P-element 26-29-$p^{KG00154}$ from Bloomington stock center #13051 (Figure S4). For *psh* rescue experiments, wild-type *pro-psh*, *pro-psh*^{M1} and *pro-psh*^{M2} were cloned in *pUAST-ATTB* vector and injected in *ATT-3B VK00033* fly lines from Bloomington stock center #24871.

Bacterial stocks

Escherichia coli (*ATCC23724*), *Enterobacter cloacae* (kind gift of H. Monteil), *Serratia marcescens* (*20C2*; described in Nehme et al., 2007), *Bacillus subtilis* (kind gift from J. Millet, Pasteur Institute of Paris) and *Erwinia carotovora* (*Ecc15*) were grown in Luria-Bertani broth (LB). *Staphylococcus aureus* (*RN6390*), *Enterococcus faecalis* (*OG1RF*), *Pseudomonas aeruginosa* (*PA14*) and *Pseudomonas entomophila* (kind gift of B. Lemaitre) were grown in Bushnell Haas Broth (BHB). *Micrococcus luteus* (*4698*) was grown in Tryptic Soy Broth (TSB). *Lactobacillus plantarum* (kind gift of WJ. Lee) was grown in deMedan, Rogosa and Sharpe broth (MRS). Fungi *Beauveria Bassiana* (*80.2*) and *Metarhizium anisopliae* (*V275*) were grown in modified TKI broth (described in Lohse et al., 2014) and *Candida glabrata* and *Candida albicans* (isolated by Pr M. Koenig, CHU Strasbourg-Hautepierre) in Sabouraud Broth. *Enterococcus faecium* and *E. faecalis gelE-* and *sprE-* null mutants are a kind gift from E. Murray (University of Texas) and have been described in Kawalec et al. (2005). *P. aeruginosa Elastase* (*ID31939*) and *Protease IV* (*ID37740*) null mutants are a kind gifts from F.M. Ausubel and are described in Liberati et al. (2006).

METHOD DETAILS

Expression of wild-type rpro-Psh, pro-Psh mutants and cathepsin 26-29-p

The coding sequence of pro-Psh was amplified by PCR using the cDNA clone *GH12385* (DGRC) as template. KpnI and XhoI sites were introduced on 5′ and 3′ respectively of the *psh* cDNA using the following primers: 5′-GGGGGGTACCAAGATGCCATT GAAGTGGTCCCTGC-3′ and 5′-GGGGCTCGAGCACCCGATTGTCCGGCCAGA-3′ with Phusion High-Fidelity (New England Biolabs) and sub-cloned into KpnI-XhoI sites (New England Biolabs) of the *pMT-V5-HisA* vector (Invitrogen). The ligated product was transformed into chemically competent *E. coli DH5*α cells (Invitrogen). Appropriate insertion of the *psh* gene into the *pMT-V5-HisA* vector was verified by DNA sequencing (GATC Biotech sequencing center). Plasmid DNA named pJM1345 was extracted from these transformed *E. coli DH5*α cells using a Plasmid purification kit (QIAGEN). Psh mutants were obtained by PCR-directed mutagenesis using pJM1345 as template and are described in Tables S1 and S2. The Psh constructs were also digested by KpnI-PmeI restriction enzymes and sub-cloned into the *pUAST-attB* vector (described in Bischof et al., 2007). Similarly, the coding sequence of cathepsin 26-29-p was amplified by PCR using the cDNA clone pRE18380 (DGRC) and primers T7/IMU1347 (Table S2) and sub-cloned into EcoRI-ApaI of the pMT-V5-His expression vector.

Immune challenge

For infection by septic injury, flies were injured with a thin tungsten needle previously dipped in a microorganism suspension diluted in PBS at the indicated concentrations. Flies were challenged by natural infection with *B. bassiana.* 18.4 nL of a solution of *B. subtilis* (P5985; Sigma-Aldrich) or *A. oryzae* (P6110; Sigma-Aldrich) proteases, diluted at (1:2000) in PBS or 9.2 nL of a sonicated suspension of *M. luteus* peptidoglycans (5mg/ml; Sigma-Aldrich) were injected into the fly body cavity (Nanoject II apparatus; Drummond Scientific). When needed, 18.4 nL of E-64 (Sigma) diluted in PBS at 0.5 or 2 mM were injected 2 hours before immune challenge.

Quantitative RT-PCR

RNA was isolated with the NucleoSpin 96 RNA Kit (Macherey-Nagel) according to the manufacturer's instructions. RNA was reversed transcribed by using the iScriptcDNA Synthesis Kit (Bio-Rad). Analysis of RNA expression was performed by real-time quantitative RT-PCR by using the iTaq SYBR Green Kit (Bio-Rad). *Ribosomal protein 49* (*Rp49*) mRNA was used for normalization. The primers were as follows: for *drs* forward, 5′-CGTGAGAACCTTTTCCAATATGATG-3′ and reverse, 5′-TCCCAGGACCACCAGCAT-3′; *Rp49* forward, 5′-GACGCTTCAAGGGACAGTATCTG-3′ and reverse, 5′-AAACGCGGTTCTGCATGAG-3′; *26-29-p* forward, 5′-CGCAG GCTTGGCTTTCTCAG-3′ and reverse, 5′- GGCGTACGGAATGTACAGGG-3′.

Azo-collagen Assay

Bacterial and fungal cultures were centrifuged for 15 min at 5,000 g. 200 μL of cell free culture supernatant were then incubated for 8 hours at 29°C under constant shacking in 0.1 M TrisHCl buffer pH 8 (final volume: 600 μl) with 0.15 mg/ml of AzoDye-collagen (Sigma). The reaction was stopped by adding 200 μL of 3 M glycine, pH 3 and the AzoDye-collagen fibers were harvested by

centrifugation (10 min at 15,000 g). The absorbance at 520 nm of the clear supernatant (200 μl) was determined in a 96-well plate using a spectrophotometer LB940 (BERTHOLD Technologies). Controls were done in the same conditions with pre-inactivated supernatant (10 min at 100°C).

Expression and purification of wild-type and mutated forms of rpro-Psh

Drosophila S2 cells were maintained at 25°C in Schneider's medium (Biowest) supplemented with 10% FCS (Thermo Scientific). A total of 2 × 10^6 S2 cells were co-transfected with each plasmid of interest (1 μg) and with puromycin-selection plasmid (0.1 μg) by calcium phosphate precipitation and selected in the presence of puromycin (0.1 μg/ml). S2 cells stably transfected were then grown in *Insect-Xpress medium* (Biowhittaker) supplemented with 1% GlutaMAX-1 (gibco) and 1% Pen Strep (gibco). Wild-type or mutant rpro-Psh expression was then induced with Cu_2SO_4 at 0.5 M for 3 days at 25°C. Cultures were harvested by centrifugation at 1,500 g for 5 min and the cell-free supernatant dialyzed 2 times against 4 L of Ni-Sepharose binding buffer (20 mM sodium phosphate buffer, 500 mM NaCl, 20 mM imidazole, pH 7.4), applied to a 10 mL pre-equilibrated Ni^{2+}-Sepharose 6 Fast Flow matrix (GE Healthcare, Pittsburgh, PA) and bound proteins were eluted with binding buffer supplemented with 500 mM imidazole. Eluted fractions containing the protein of interest were pooled and dialyzed against 0.1 M Tris-buffer pH 8. Protein concentrations of the final samples were determined by BCA Assay (Sigma).

Hydrolysis assay of rpro-Psh

Bacteria and fungi were grown to early stationary phase and centrifuged (5,000 g, 15 min). The cellular fraction was diluted in an equal volume of PBS and the cell-free medium was concentrated 10 times (CorningR Spin-X UF concentrators, Sigma). 20 μL of purified rpro-Psh (0.4 μg/μl in 0.1 M Tris buffer, pH 8) was incubated with 20 μL of the various microbial preparations. After 1 or 4 hours at 29°C, 1 μg of rpro-Psh was taken and the reaction was stopped at 100°C for 5 min in the presence of NuPAGE LDS Sample buffer (Invitrogen). Samples were electrophoresed for 2 hours at 100 V on a NuPAGE 4%–12% Bis-Tris Gel (Invitrogen) and resolved proteins electro-blotted onto nitrocellulose membrane (2 hours at 30 V). Non-specific binding sites were blocked with a 5% skim milk solution and membranes were then incubated with the monoclonal Anti-6His C-term antibody (Invitrogen) followed by the anti-mouse IgG-peroxidase conjugate (Sigma). Proteins of interest were visualized with the Chimioluminescent Reagent substrate Covalight (Covalab).

Alternatively, rpro-Psh was incubated under the same conditions with microbial cell-free medium. At various time points, 5 μg of proteins were removed and the reaction was stopped at 100°C for 5 min in the presence of NuPAGE LDS Sample buffer and subjected to SDS-PAGE electrophoresis as above. Proteins were then stained with SimplyBlue SafeStain (Invitrogen) and N-terminal extremities of the hydrolysis products of interest were determined by N-terminal labeling and mass spectrometry (see below).

Activity assay of rpro-Psh

Cell free supernatant of S2 cells expressing rPro-Psh (200 μl) was incubated in 0.1 M Tris buffer, pH 8 with *B. subtilis* protease (1 nM), with *A. oryzae* protease (100 nM) or with 200 μL of cell free medium of microbial culture (final volume: 600 μl). After 1 hour, proteolytic activity of the generated rpro-Psh hydrolysis products was determined on the fluorogenic substrate Z-Arg-AMC (Sigma Aldrich) for 30 min at 29°C in 0.1 M Tris buffer pH 8 supplemented with 5 mM $CaCl_2$ (λ_{ex} = 350 nm; λ_{em} = 460 nm).

Expression and activation of rpro-cathepsin 26-29-p

S2 cells were stably transfected with the expression plasmid of rpro-cathepsin 26-29-p as described previously. Expression of rpro-cathepsin 26-29-p was assessed in S2 cells lysate and cells culture supernatant by western blot using the monoclonal Anti-6His C-term antibody. To activate rpro-cathepsin 26-29-p, cells culture media was concentrated 20 times on Amicon centrifugal filter (Millipore) and then incubated with pepsine (0.002 mg/ml) in 0.1 M glycine buffer, pH 3. After incubation at 37°C, rpro-cathepsin 26-29-p processing was followed by western blot with the monoclonal Anti-6His C-term antibody. Activity of the generated hydrolysis products was assessed at 37°C in 0.1 M sodium acetate buffer, ph 5.5 on the fluorogenic substrate Z-Phe-Arg-AMC (Bachem) (λ_{ex} = 350 nm; λ_{em} = 460 nm).

In vitro sequential activation of rpro-Psh

Purified rpro-Psh was incubated as previously described in 0.1 M Tris buffer, pH 8 with or without *E. faecalis* culture supernatant at 29°C. After 3 hours, the partially processed rpro-Psh was incubated with the pre-activated cathepsin 26-29-p for 30 min to 2 hours at 29°C in 0.2 M sodium acetate buffer, pH 5.5. The generated hydrolysis products were then visualized after SDS-PAGE electrophoresis using the SimplyBlue SafeStain (Invitrogen) and N-terminal extremities of the hydrolysis products of interest were determined by N-terminal labeling and mass spectrometry (see below). Controls were performed in presence of E-64 (0.2 mM). To confirm the capacity of cathepsin 26-29-p to release the N-terminal extremity of the active form of Psh, the experiment was repeated in the same conditions with rpro-Psh His143/Glu.

In-Gel N-Terminal Protein Derivatization strategy

Unless otherwise specified, all chemicals were obtained from Sigma Aldrich (St. Louis, MO). Using an automated robot platform (Massprep station, Waters), the gel slices containing protein samples were washed twice in 25 mM NH_4HCO_3 and CH_3CN. The

cysteine residues where subsequently reduced in 10 mM (tris(2-carboxyethyl)phosphine) at room temperature and then alkylated with 30 mM iodoacetamide. After dehydration with CH_3CN, (N-succinimidyloxycarbonyl-methyl)tris-(2,4,6-trimethoxyphenyl)phosphonium bromide (TMPP) was added at a molar ratio of 200:1 (quantities of protein were evaluated based on 1D gel intensity band). Then 50 μL of the reaction buffer (100 mM Tris-HCl, pH 8.2) was added in each well (1H). Selective N-terminal TMPP derivatization is achieved by a careful control of reaction pH at 8.2. Residual derivatizing reagent was quenched by adding a solution of 0.1 M hydroxylamine at room temperature for 1 hour. The gel slices were then washed three times in 25 mM NH_4HCO_3 and CH_3CN before dehydration with CH_3CN. Enzymatic digestion was performed in-gel overnight at 37°C using porcine trypsin (Promega, Madison, WI, USA). Peptides were suspended in 10 μL of 1% CH_3CN, 0.1% HCO_2H in H_2O.

Peptides were analyzed on a nanoUPLC-system (nanoAcquity, Waters) coupled to a quadrupole-Orbitrap hybrid mass spectrometer (Q-Exactive Plus, Thermo Scientific, San Jose, CA). Sample was concentrated/desalted on a Symmetry C18 precolumn (0.18 × 20 mm, 5 μm particle size; Waters) using a mobile phase composed of 99% solvent A (0.1% HCO_2H in H_2O) and 1% solvent B (0.1% HCO_2H in CH_3CN) at a flow rate of 5 μl/min for 3 minutes. Afterward, peptides were eluted at a flow rate of 450 nL/min using a UPLC separation column (BEH130 C18, 200 mm x 75 μm, 1.7 μm particle size; Waters) maintained at 60°C with the following gradient: from 1% to 50% B in 50 minutes.

The Q-Exactive Plus was operated in positive ion mode with source temperature set to 250°C and spray voltage to 2.0 kV. Spectra were acquired through automatic switching between full MS and MS/MS scans. Full scan MS spectra (300-1800 m/z) were acquired at a resolution of 70,000 at m/z 200 with an automatic gain control (AGC) fixed at 3×10^6 ions and a maximum injection time set to 50 ms, the lock-mass option being enabled (polysiloxane, 445.12002 m/z). Up to 10 most intense precursors (with a minimum of 2 charges) per full MS scan were isolated using a 2 m/z window and MS/MS spectra were acquired at a resolution of 17,500 at m/z 200 with an AGC fixed at 1×10^5 and a maximum injection time set to 100 ms. Peptide fragmentation was performed using higher energy collisional dissociation (HCD), with normalized collision energy being set to 27 and dynamic exclusion of already fragmented precursors being set to 10 s. The peptide match selection option was turned on. The system was fully controlled by XCalibur software (v3.0.63; Thermo Fisher Scientific).

Peak lists in MGF format were generated using the MSConvert algorithm of ProteoWizard software (v3.0.6090; http://proteowizard.sourceforge.net/). Searched against a SwissProt protein database combining *Drosophila melanogaster* (TaxID 7227) using Mascot algorithm v2.5.1 (Matrix science, London, UK). Mass tolerance was set to 5 ppm in MS mode and 0.07 Da in MS/MS mode, a maximum of one trypsin-missed cleavage was tolerated. Oxidation of methionine residues and carbamidomethylation of cysteine residues and TMPP of N-terminal peptide were considered as variable modifications. To gather and validate the identifications obtained, Scaffold software was used and spectra of labeled peptides were then carried out for manual validation (parameters Ion Score ≥ 0 and Ion -Identity Score = -40).

QUANTIFICATION AND STATISTICAL ANALYSIS

GraphPad Prism 05 (GraphPad Software) was used for mean and standard error calculations and Student's t test or One-way ANOVA test. Log-rank analyses of survival assay were performed with the OASIS online application (Yang et al., 2011).

DATA AND SOFTWARE AVAILABILITY

Original images of gels and blots have been deposited to Mendeley Data and are available at https://doi.org/10.17632/mzgnrcftzv.1.

Molecular Cell, Volume *69*

Supplemental Information

The Circulating Protease Persephone Is an Immune Sensor for Microbial Proteolytic Activities Upstream of the *Drosophila* Toll Pathway

Najwa Issa, Nina Guillaumot, Emilie Lauret, Nicolas Matt, Christine Schaeffer-Reiss, Alain Van Dorsselaer, Jean-Marc Reichhart, and Florian Veillard

Figures S1 to S5

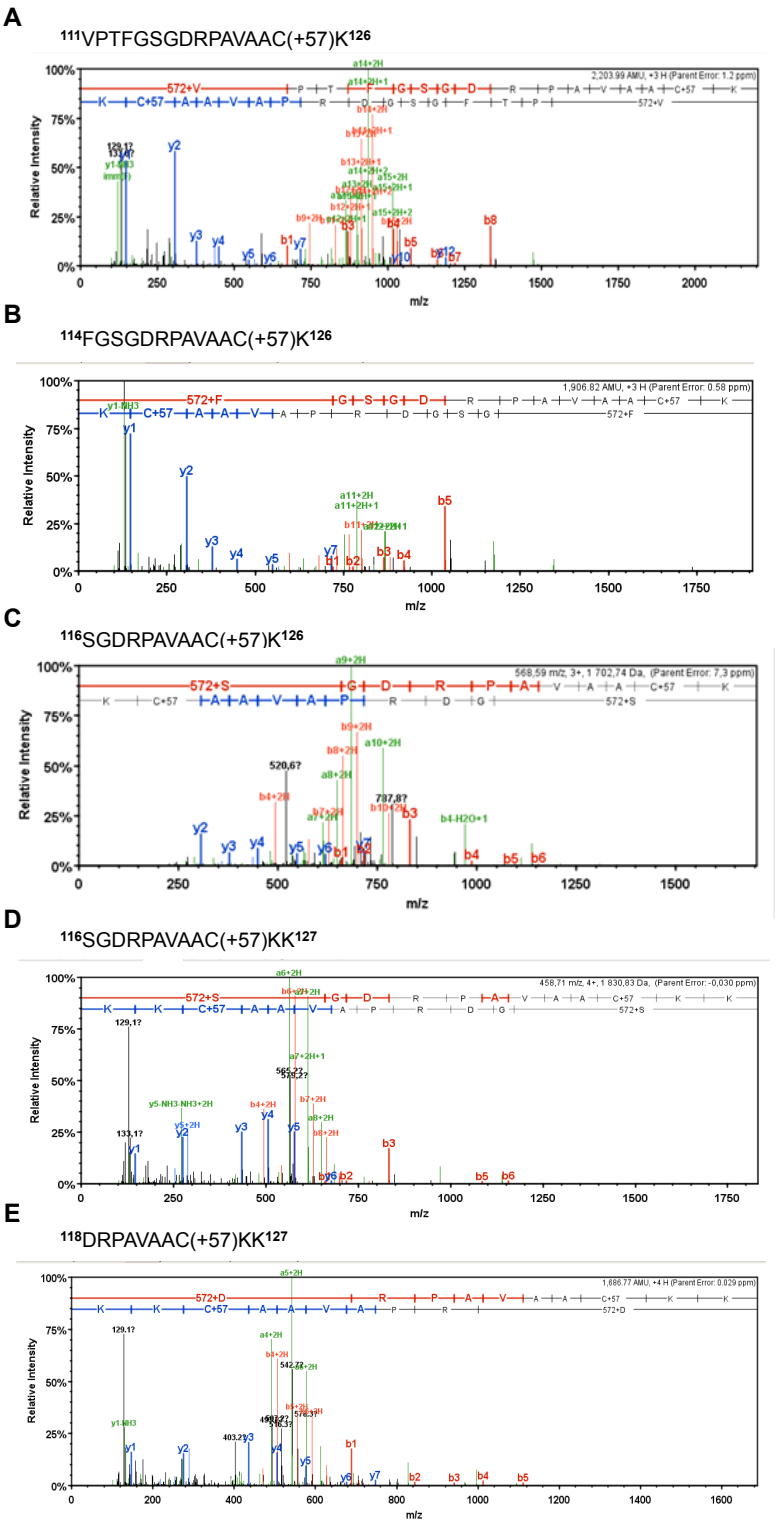

Figure 1 : MS/MS spectra of the N-terminal peptides identified using in-gel protein strategy after proteolytic maturation (Related to Figure 3)
(A) *E. faecalis* and *S. marcescens*, (B) *P. aeruginosa*, (C) *E. faecalis*, (D) *S. aureus* and *B. bassiana* and (E) *S. aureus*.

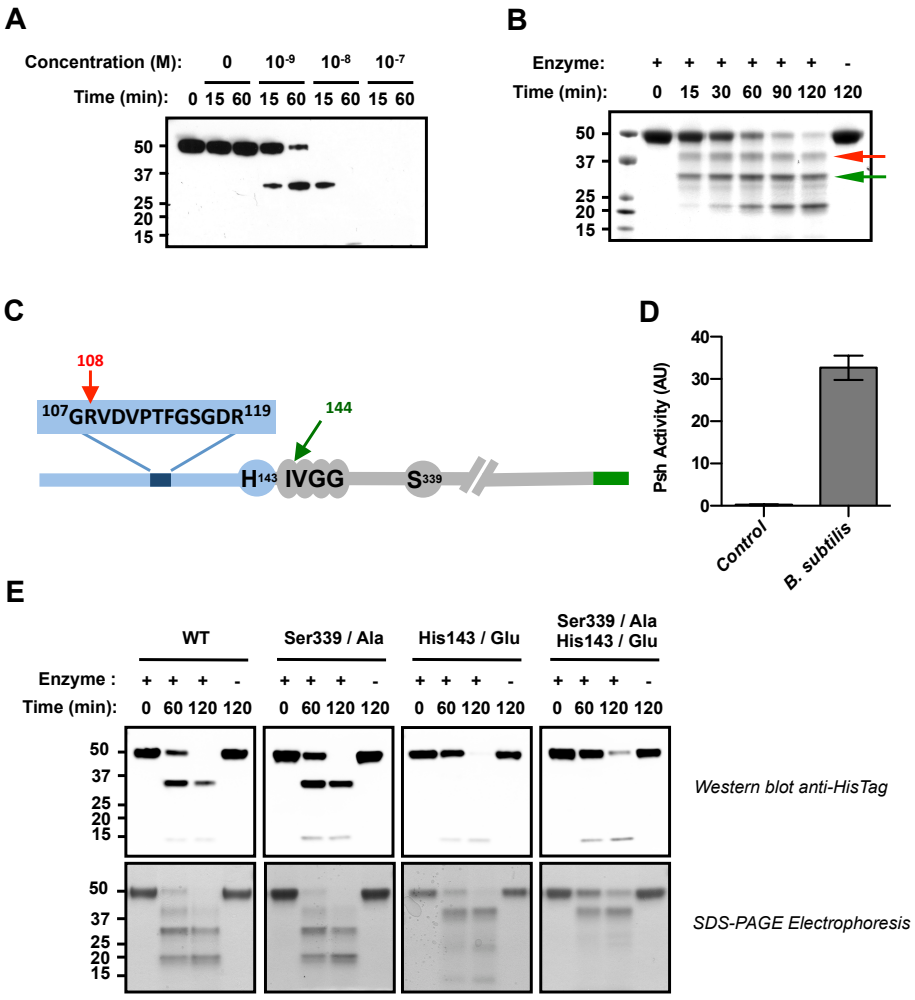

Figure S2: Specific activation of Psh by *B. subtilis* subtilisin (Related to Figure 3)

(A) rpro-Psh (0.2 µg/µl) was incubated at 29 °C with various concentrations of *B. subtilis* purified subtilisin. After 15 or 60 min, 1 µg aliquots were removed and boiled for 5 min to stop the reaction. After SDS-PAGE electrophoresis, residual rpro-Psh was visualized using anti-HisTag antibody. (B) rpro-Psh (0.2 µg/µl) was incubated at 29 °C with *B. subtilis* (1 nM) purified protease. At various time points, 5 µg aliquots were removed and boiled for 5 min to stop the reaction. Samples were then electrophoresed and stained with Coomassie blue. Representative results of at least 2 independent experiments. (C) The N-terminal extremities of the main hydrolysis products (indicated by arrows) were determined by nanoLC-MS/MS analysis after in-gel protein N-terminal labeling using TMPP-Ac-Osu. (D) Cell-free supernatant of S2 cells expressing rpro-Psh (200 µl) was incubated in TrisHCl buffer 0.1 M, pH 8 with *B. subtilis* (1 nM) protease. After 1 hour, proteolytic activity of the generated rPro-Psh hydrolysis products was determined on the fluorogenic substrate Z-Arg-AMC for 30 min at 29 °C in 0.1 M TrisHCl buffer pH 8 supplemented with 5 mM $CaCl_2$. (E) rpro-Psh mutants His143/Glu, Ser339/Ala and His143/Glu; Ser339/Ala (0.2 µg/µl) were incubated with *B. subtilis* protease under the same conditions. After 1 or 2 hours, residual proteins were observed by Western blot with anti-6HisTag antibody and hydrolysis products visualized by Coomassie blue staining.

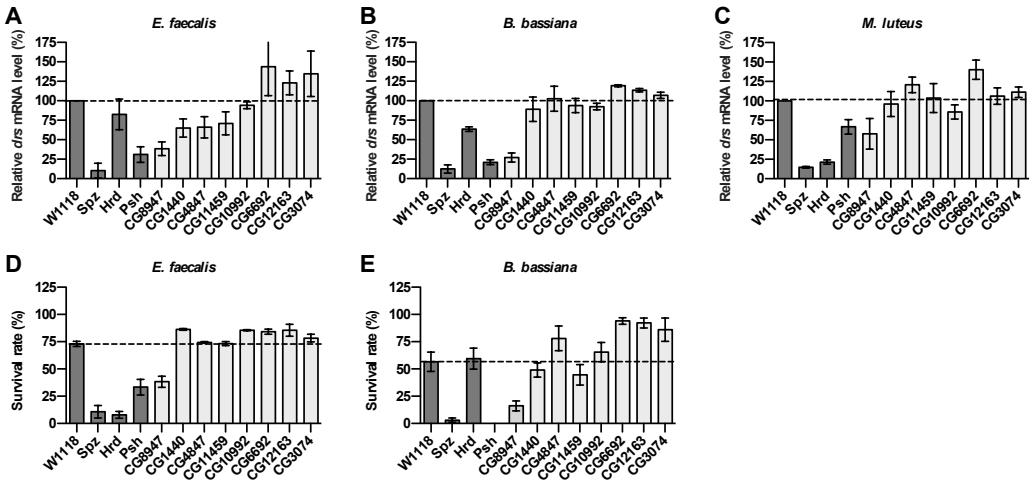

Figure S3: Implication of cysteine cathepsins in the Toll pathway (Related to Figure 5)

(A-C) Flies deficient for the indicated gene (See Sup Table 1) were challenged by septic injury with *E. faecalis* (OD_{600}=1) (A), *M. luteus* (OD_{600}>200) (C) or by natural infection with *B. bassiana* (B). After 24 hours at 29 °C, flies were collected and *drs* gene expression was monitored by RT-qPCR in total RNA extracts. *Ribosomal protein 49* (*Rp49*) mRNA was used as reference gene. Results were normalized to the value obtained for w^{1118} control flies. Data represent means ± standard errors of 3 independent experiments, each containing three groups of 10 flies (5 males and 5 females). (D-E) Survival rate of adult flies challenged with *E. faecalis* by septic injury (OD_{600}=1) or with *B. bassiana* by natural infection 72 hours post-infection. Results are normalized with control flies (w^{1118} flies for null mutants and *C564-gal4* flies for RNAi expressing flies). Data represent means ± standard errors of 3 independent experiments, each containing three groups of 20 flies (10 males and 10 females).

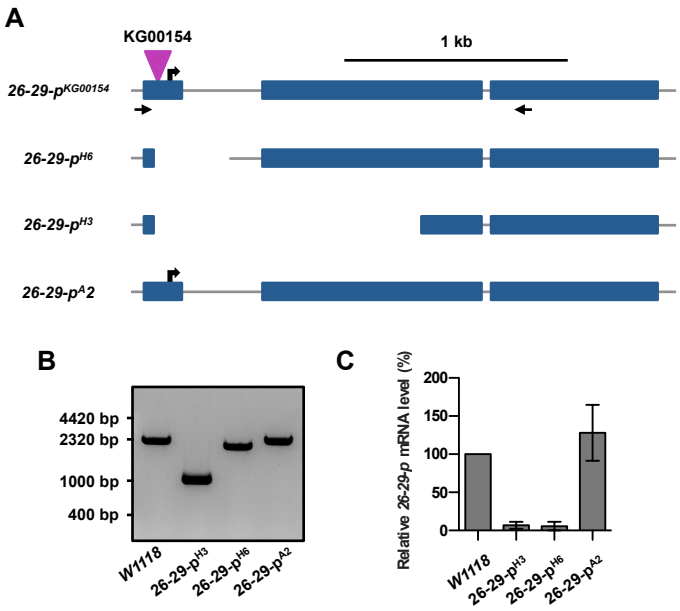

Figure S4: Generation of cathepsin 26-29-p mutants (Related to Figure 6)
(A) Schematic representation of the genomic region of *26-29-p* and the mutant alleles obtained by P-element excision. Two null mutants and one revertant fly lines were generated by excision of the *KG00154* P-element (purple arrow) following crosses with *P(Δ2-3)* transposase flies (Bloomington #2534). (B) PCR products obtained using the primers forward: 5'-GTCCGACTATCGGTTCGGTTT-3' and reverse: 5'-GATTGCCGCCATTCTTCAGG-3' and indicated by black arrows in (A). (C) Flies were collected and *26-29-p* gene expression was monitored by RT-qPCR in total RNA extracts. *Ribosomal protein 49* (*Rp49*) mRNA was used for normalization. Data represent means ± standard errors of 3 independent experiments, each containing three groups of 10 flies (5 males and 5 females).

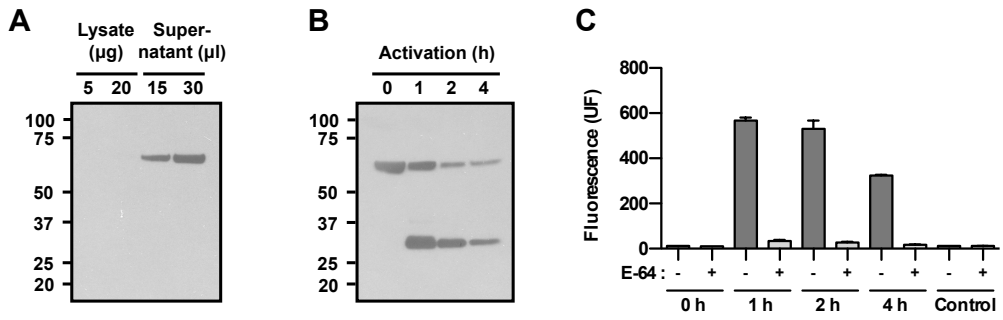

Figure S5: Expression and activation of rpro-cathepsin 26-29-p (Related to Figure 7)

S2 cells were stably transfected with the expression plasmid of rpro-cathepsin 26-29-p as described previously. (A) Expression of rpro-cathepsin 26-29-p was assessed in S2 cells lysate and cells culture supernatant by Western blot using the monoclonal Anti-6His C-term antibody. (B) To activate rpro-cathepsin 26-29-p, cells culture media was concentrated 20 times and then incubated with pepsine (0.002 mg/ml) in 0.2 M Glycine buffer, pH 4. After incubation at 37 °C, rpro-cathepsin 26-29-p processing was followed by Western blot with the monoclonal Anti-6His C-term antibody. (C) Activity of the generated hydrolysis products was assessed with or without E-64 (0.1 mM) at 37 °C in 0.1 M sodium acetate buffer, pH 5.5 on the fluorogenic substrate Z-Phe-Arg-AMC (λ_{ex} = 350 nm; λ_{em} = 460 nm). Pepsine alone was used as control.

Supplementary Table S1 TO S5

Table S1: List of Plasmids (Related to STAR Methods)

Plasmid name	Construction	Destination plasmid	Details
pJM1345	rpro-Psh	pMT-V5	PCR on cDNA clone GH12385 (DGRC) with primers IMU839/840 cloned in KpnI-XhoI
pJM1681	rpro-Psh^{M1}	pMT-V5	PCR on pJM1345 with primers IMU1144/1342, IMU1341/1145, IMU1144/1145 cloned in BglII-XhoI
pJM1682	rpro-Psh^{M2}	pMT-V5	PCR on pJM1345 with primers IMU 1343/1344 cloned in Bgl2-XhoI
pJM1692	pro-Psh	pUAST-ATTB	KpnI-PmeI fragment from pJM1345
pJM1693	pro-Psh^{M1}	pUAST-ATTB	KpnI-PmeI from fragment pJM1681
pJM1694	pro-Psh^{M2}	pUAST-ATTB	KpnI-PmeI fragment from pJM1682
pJM1674	rpro-Psh Ser339 / Ala	pMT-V5	PCR on pJM1345 with primers IMU1144/1228, IMU1145/1229, IMU1144/1145 cloned in KpnI-XhoI
pJM1675	rpro-Psh His144 / Glu	pMT-V5	PCR on pJM1345 with primers IMU1144/1338, IMU1145/1337, IMU1144/1145 cloned in KpnI-XhoI
pJM1676	rpro-Psh His143 / Glu ; Ser339 / Ala	pMT-V5	PCR on pJM674 with primers IMU1144/1338, IMU1145/1337, IMU1144/1145 cloned in KpnI-XhoI
pJM1696	rpro-cathepsin 26-29-p	pMT-V5	PCR on cDNA clone pRE 18380 (DGRC) with primers T7/IMU1347 cloned in EcoR1-Apa1

Table S2: List of Primers for PCR (Related to STAR Methods)

Primer name	Sequence (5'-3')
IMU 839	GGGGGGTACCAAGATGCCATTGAAGTGGTCCCTGC
IMU 840	GGGGCTCGAGCACCCGATTGTCCGGCCAGA
IMU 1144	TGTGGTCAGCAGCAAAATCAAGTG
IMU 1145	CTGCATTCTAGTTGTGGTTTGTCC
IMU 1228	GCTCATGAATGAGCGGCCCACCGGCGTCGCCCTTGCATGCGTCGGCG
IMU 1229	CGCCGACGCATGCAAGGGCGACGCCGGTGGGCCGCTCATTCATGAGC
IMU 1337	GAGCGGCAATCAATTGGTCATAGACATCGTGGGCGGTTATCC
IMU 1338	GGATAACCGCCCACGATGTCTATGACCAATTGATTGCCGCTC
IMU 1341	GCTGCTGCTGCTGCTCCCACGTTCGGAAGCGGT
IMU 1342	AGCAGCAGCAGCAGCACTGGTCATTGGAGCTTTTGTGC
IMU 1343	GCTGCTGCTGCTGCTGCTGCTGCTAGCGGTGATCGCCCAGC
IMU 1344	AGCAGCAGCAGCAGCAGCAGCAGCACTGGTCATTGGAGCTTTTGTGC
IMU 1345	GCACAAAAGCTCCAATGACCAGTAGCGGTGATCGCCCAGC
IMU 1346	GCTGGGCGATCACCGCTACTGGTCATTGGAGCTTTTGTGC
IMU 1347	GCTTACCTTCGAAGGGCCCCATCTCCACATAAGTGGGCATGG
T7	TGTAAAACGACGGCCAGTGA

Table S3: Screening of cysteine cathepsins (Related to STAR Methods)

Name	CG number	Bloomington stock number	Genotype
26-29-p	CG8947	13051	P(SUPor-P)26-29-p^{KG00154}
Cathepsin L1	CG6692	32932	P(TRIP.HMS00725)attP2
CG4847	CG4847	42655	P(TRIP.HMS02491)attP2
CG12163	CG12163	33955	P(TRIP.HMS00910)attP2
CG11459	CG11459	50488	Mi(MIC)CG11459^{MI08810}
Cathepsin B1	CG10992	15434	P(EPgy2)CtsB1^{EY03339}
Swing	CG3074	36591	P(TRIP.GL00551)attP2
Bleomycin Hydrolase	CG1440	13977	P(SUPor-P)CG1440^{KG04580}

Table S4: Ms/Ms fragmentation table of the N-terminal TMPP labeled peptide IVGGYPVDPGVYPHMAAIGYITFGTDFR (Mascot interpretation, Related to Figure 3).

amino acid	b ion	a (M)	a* (M)	a*++ (M)	a++ (M)	a0 (M)	a0++ (M)	b (M)	b++ (M)	b0 (M)	b0++ (M)	y (M)	y* (M)	y*++ (M)	y++ (M)	y0 (M)	y0++ (M)	y ion	
I	1	658.2776	641,251	321,1291	329,6424	640,267	320,6371	686.2725	343,6399										28
V	2	757.346	740,3194	370,6634	379,1766	739,3354	370,1713	785.3409	393,1741			2916,403	2899,376	1450,192	1458,705	2898,392	1449,7		27
G	3	814,3674	797,3409	399,1741	407,6874	796,3569	398,6821	842,3624	421,6848			2817,334	2800,308	1400,657	1409,171	2799,324	1400,165		26
G	4	871.3889	854,3624	427,6848	436,1981	853,3783	427,1928	899.3838	450,1955			2760,313	2743,286	1372,147	1380,66	2742,302	1371,655		25
Y	5	1034.4522	1017,426	509,2165	517,7298	1016,442	508,7245	1062.4471	531,7272			2703,291	2686,265	1343,636	1352,149	2685,281	1343,144		24
P	6	1131,505	1114,478	557,7429	566,2561	1113,494	557,2509	1159,5	580,2536			2540,228	2523,201	1262,104	1270,618	2522,217	1261,612		23
V	7	1230.5734	1213,547	607,2771	615,7903	1212,563	606,7851	1258.5683	629,7878			2443,175	2426,149	1213,578	1222,091	2425,165	1213,086		22
D	8	1345,6	1328,574	664,7905	673,3038	1327,59	664,2985	1373.5953	687.3013	1355,585	678,296	2344,107	2327,08	1164,044	1172,557	2326,096	1163,552		21
P	9	1442,653	1425,627	713,3169	721,8302	1424,643	712,8249	1470,648	735,8277	1452,638	726,8224	2229.0797	2212,053	1106,53	1115.0435	2211,069	1106,038		20
G	10	1499,675	1482,648	741,8277	750,3409	1481,664	741,3356	1527.6695	764,3384	1509,659	755,3331	2132.027	2115	1058,004	1066,517	2114,016	1057,512		19
V	11	1598,743	1581,716	791,3619	799,8751	1580,732	790,8699	1626.7379	813,8726	1608,727	804,8673	2075,006	2057,979	1029,493	1038,006	2056,995	1029,001		18
Y	12	1761,806	1744,78	872,8935	881,4068	1743,796	872,4015	1789.8012	895,4043	1771,791	886,399	1975.9371	1958,911	979,9589	988.4722	1957,927	979,4669		17
P	13	1858,859	1841,833	921,4199	929,9332	1840,849	920,9279	1886,854	943,9306	1868,843	934,9254	1812.8738	1795,847	898,4272	906,9405	1794,863	897,9352		16
H	14	1995,918	1978,891	989,9494	998,4626	1977,907	989,4574	2023,913	1012,46	2005,902	1003,455	1715,821	1698,795	849,9009	858,4141	1697,81	849,4089		15
M	15	2142,953	2125,927	1063.4671	1071,98	2124,943	1062,975	2170,948	1085,978	2152,938	1076,973	1578.7621	1561,736	781,3714	789,8847	1560,752	780,8794		14
A	16	2213,991	2196,964	1098,986	1107,499	2195.9799	1098,494	2241.9854	1121,496	2223,975	1112,491	1431.7267	1414,7	707,8537	716,367	1413,716	707,3617		13
A	17	2285,028	2268,001	1134,504	1143,018	2267,017	1134,012	2313,023	1157,015	2295,012	1148,01	1360.6896	1343,663	672,3352	680,8484	1342,679	671,8431		12
I	18	2398,112	2381,085	1191,046	1199,56	2380,101	1190,554	2426,107	1213,557	2408,096	1204,552	1289.6525	1272,626	636,8166	645,3299	1271,642	636,3246		11
G	19	2455,133	2438,107	1219,557	1228,07	2437,123	1219,065	2483,128	1242,068	2465,118	1233,062	1176.5684	1159,542	580,2746	588,7878	1158,558	579,7826		10
Y	20	2618,197	2601,17	1301,089	1309,602	2600,186	1300,597	2646,191	1323,599	2628,181	1314,594	1119.5469	1102,52	551,7638	560,2771	1101,536	551,2718		9
I	21	2731,281	2714,254	1357,631	1366,144	2713,27	1357,139	2759,276	1380,141	2741,265	1371,136	956.4836	939,4571	470,2322	478,7454	938,473	469,7402		8
T	22	2832,328	2815,302	1408,155	1416,668	2814,318	1407,663	2860,323	1430,665	2842,313	1421,66	843.3995	826,373	413,6901	422,2034	825.389	413,1981		7
F	23	2979,397	2962,37	1481,689	1490,202	2961,386	1481,197	3007,392	1504,199	2989,381	1495,194	742.3519	725,3253	363,1663	371,6796	724,3413	362,6743		6
G	24	3036,418	3019,392	1510,199	1518,713	3018,408	1509,707	3064,413	1532,71	3046,403	1523,705	595.2835	578,2569	289,6321	298,1454	577,2729	289,1401		5
T	25	3137,466	3120,439	1560,723	1569,237	3119,455	1560,231	3165,461	1583,234	3147,45	1574,229	538.262	521,2354	261,1214	269,6346	520,2514	260,6293		4
D	26	3252,493	3235,466	1618,237	1626.75	3234,482	1617,745	3280,488	1640,748	3262,477	1631,742	437.2143	420,1878	210,5975	219,1108	419,2037	210,1055		3
F	27	3399,561	3382,535	1691,771	1700,284	3381,551	1691,279	3427,556	1714,282	3409,546	1705,276	322.1874	305.1608	153,084	161,5973				2
R	28											175.119	158,0924	79,5498	88,0631				1

Table S5: Log-rank analyses of flies survival assays (OASIS online application) (Related to Figure 6)

	B. bassiana			*E. faecalis*		
Condition	**Chi^2**	**P-value**	**Bonferroni P-value**	**Chi^2**	**P-value**	**Bonferroni P-value**
w^{1118} v.s. Psh	78.36	0.0e+00	0.0e+00	16.63	4.5e-05	0.0002
w^{1118} v.s. Spz	79.56	0.0e+00	0.0e+00	62.94	0.0e+00	0.0e+00
w^{1118} v.s. 26-29-p^{H3}	84.16	0.0e+00	0.0e+00	14.72	0.0001	0.0006
w^{1118} v.s. 26-29-p^{H6}	67.11	0.0e+00	0.0e+00	9.70	0.0018	0.0092
w^{1118} v.s. 26-29-p^{A2}	1.83	0.1758	0.8792	0.00	0.9505	1.0000
Psh v.s. w^{1118}	78.36	0.0e+00	0.0e+00	16.63	4.5e-05	0.0002
Psh v.s. Spz	5.32	0.0211	0.1056	32.07	1.5e-08	7.5e-08
Psh v.s. 26-29-p^{H3}	2.52	0.1122	0.5610	0.01	0.9195	1.0000
Psh v.s. 26-29-p^{H6}	2.34	0.1261	0.6304	0.65	0.4214	1.0000
Psh v.s. 26-29-p^{A2}	49.70	0.0e+00	0.0e+00	17.63	2.7e-05	0.0001
Spz v.s. w^{1118}	79.56	0.0e+00	0.0e+00	62.94	0.0e+00	0.0e+00
Spz v.s. Psh	5.32	0.0211	0.1056	32.07	1.5e-08	7.5e-08
Spz v.s. 26-29-p^{H3}	0.18	0.6751	1.0000	24.51	7.4e-07	3.7e-06
Spz v.s. 26-29-p^{H6}	8.90	0.0028	0.0142	29.92	4.5e-08	2.3e-07
Spz v.s. 26-29-p^{A2}	54.91	0.0e+00	0.0e+00	66.53	0.0e+00	0.0e+00
26-29-p^{H3} v.s. w^{1118}	84.16	0.0e+00	0.0e+00	14.72	0.0001	0.0006
26-29-p^{H3} v.s. Psh	2.52	0.1122	0.5610	0.01	0.9195	1.0000
26-29-p^{H3} v.s. Spz	0.18	0.6751	1.0000	24.51	7.4e-07	3.7e-06
26-29-p^{H3} v.s. 26-29-p^{H6}	6.96	0.0083	0.0416	0.61	0.4351	1.0000
26-29-p^{H3} v.s. 26-29-p^{A2}	55.06	0.0e+00	0.0e+00	15.56	0.0001	0.0004
26-29-p^{H6} v.s. w^{1118}	67.11	0.0e+00	0.0e+00	9.70	0.0018	0.0092
26-29-p^{H6} v.s. Psh	2.34	0.1261	0.6304	0.65	0.4214	1.0000
26-29-p^{H6} v.s. Spz	8.90	0.0028	0.0142	29.92	4.5e-08	2.3e-07
26-29-p^{H6} v.s. 26-29-p^{H3}	6.96	0.0083	0.0416	0.61	0.4351	1.0000
26-29-pH6 v.s. 26-29-p^{A2}	39.74	0.0e+00	0.0e+00	10.51	0.0012	0.0059
26-29-p^{A2} v.s. w^{1118}	1.83	0.1758	0.8792	0.00	0.9505	1.0000
26-29-p^{A2} v.s. Psh	49.70	0.0e+00	0.0e+00	17.63	2.7e-05	0.0001
26-29-p^{A2} v.s. Spz	54.91	0.0e+00	0.0e+00	66.53	0.0e+00	0.0e+00
26-29-p^{A2} v.s. 26-29-p^{H3}	55.06	0.0e+00	0.0e+00	15.56	0.0001	0.0004
26-29-p^{A2} v.s. 26-29-p^{H6}	39.74	0.0e+00	0.0e+00	10.51	0.0012	0.0059

VIII. Conclusion and discussion

Since its introduction to modern biology in the early years of the 20th century, the fruit fly *Drosophila melanogaster* has been one of the most extensively studied organisms. It serves as a biological model system for higher eukaryotes, including humans. *Drosophila* research has been at the forefront of many areas of biology, from genetics to molecular evolution, neurobiology, immunology, developmental biology, and cell biology. The complete nucleotide sequence of the *Drosophila* genome was determined and annotated (Adams et al., 2000). The sequence data greatly facilitated the functional studies of the ≈ 1.4 x 10^4 genes found in the fruit fly genome. Since the discovery of antimicrobial peptide responses around 40 years ago, *Drosophila* has proven to be a powerful model for the study of innate immunity (Strominger, 2009). Early studies focused on innate immune mechanisms of microbial recognition and subsequent NF-κB signal transduction. More recently, *D. melanogaster* has been employed to understand how the immune system is regulated and coordinated at the systemic level. Studies in flies can reveal how immunity and its deregulation can affect whole-body pathophysiology. Since the initial discovery of the Toll pathway in *Drosophila* development more than 30 years ago, research in the field has firmly established the role of Toll signaling in immunity as well. In the late 1980s and early 1990s two theories were suggested to consider the activation of the innate immune system by a restricted number of receptors. Heralded by C. Janeway and P. Matzinger, these theories reported that the innate immune reponse is triggered by specific non-self molecules and danger signals, respectively (Janeway, 1989; Matzinger, 1994). Interestingly, the Toll pathway in *Drosophila* is a convergence point for these two theories in the way that sensing of infection by circulating PRRs as well as detection of virulence factors realeased by the invading microorganisms by the Clip-SP Psh, both culminate in the proteolysis-dependent activation of Spz in the hemolymph upon infection.

Clip-SPs are extracellular circulating SPs found specifically in Arthropods. The powerful genetic tools available for *D. melanogaster* had been crucial to determine the range of biological processes in which Clip-SPs participate. First,

the role of 4 SPs (Snk, Ea, Gd and Ndl) in the activation of Spz during embryogenesis was elucidated at the end of the 80's, before the Clip-domain was identified. The implication of Clip-SPs in the activation of the Toll pathway during immune responses has been investigated more recently; however, various facets of this response still need to be elucidated. The proteolytic cascade triggered upon activation of the circulating receptors seems to have several missing elements. Besides, in agreement with its chymotrypsin-like activity, ModSP is theorically unable to activate Grass *in vitro* because its activation site includes an Arg residue in P1 position (Buchon et al., 2009). Furthermore, a large-scale *in vivo* RNAi screen identified the Clip-SP Spirit and the Clip-SPHs Sphinx1/2 and Spheroide as candidates potentially involved in the Toll pathway (Kambris et al., 2006). Spheroide was shown to participate in sensing of pathogenic Gram-positive bacteria (Patrnogic and Leclerc, 2017); however its exact role as well as the role of Spirit and Sphinx1/2 remains ambiguous.

Moreover, the proteolytic pathways leading to the activation of PPO in *Drosophila* are far from being understood. Probably several redundant or parallel pathways co-exist and need to be decoded. The isolation of proteases for further *in vitro* molecular studies is hampered by the small size of *Drosophila* adults and the modest amount of material that can be extracted. Insects of larger size and of easily extractable hemolymph, such as *Bombyx mori*, *Manduca sexta*, and *Tenebrio molitor*, are better choices for biochemical and structural characterization. Our knowledge of the *Drosophila* proteolytic cascades is incomplete; they may be involved in other physiological responses and signaling cascades. The role of Clip-SPHs remains widely unexplored in *Drosophila*, although they may act as cofactors for active SPs. This hypothesis has been confirmed so far only for the Clip-SPH *Hd*-PPAF-II, which binds and activates PO (Kim et al., 2002). Structural and biochemichal characterization of *Drosophila* Clip-SPs with undefined function could help to identify new cofactors implied in the immune response, to attribute a role to Clip-SPHs, and more generally could lead to a better understanding of the sequential organization and regulation of these proteolytic cascades.

Besides, up to now it has been unclear how the Toll pathway could detect a

broad spectrum of virulence factors, which often target specific host molecules. This is particularly true for pathogens proteases due to their high enzymatic specificities. While it has been previously proposed that Psh could be directly activated by microbial proteases (Gottar et al., 2006), the precise cleavage needed to activate Psh seemed to be in contradiction with its function as a broad sensor of microbial proteases. In our study, we demonstrated that Psh is able to directly sense exogenous proteases secreted by invading microorganisms (Issa et al., 2018). The study allowed us to identify in the Clip-domain of Psh a critical region that is highly sensitive to proteolysis and functioning as "bait" for microbial proteases, regardless of their specificity, origin or type. Interestingly, we showed that Gram-negative bacteria such as *Pseudomonas aeruginosa* or *Serratia marcescens*, excreting proteolytic virulence factors are able to activate the Psh-dependent Toll pathway. This seems to be a breach of the dogma (Toll *vs*. IMD) for immune response activation.

We could show how Psh plays the role of an innate immune receptor able to mechanistically sense a broad variety of microbes through virulence factors rather than specific molecular patterns (PAMPs) (Issa et al., 2018). Although we concentrated our study on exogenous proteases, we can speculate that the lack of specificity of Psh activation process allows the detection of ectopic concentrations of endogenous proteases in the hemolymph. Indeed, overexpressions of Grass in the hemolymph (El Chamy et al., 2008), as well as necrosis induction in flies (Ming et al., 2014) lead both to a Psh-dependent activation of the Toll pathway. Psh is activated through a sequential mechanism including an initial cleavage within the sensitive "bait-region" licensing the subsequent maturation of Psh to the endogenous cysteine cathepsin 26-29-p. Psh is then able to activate SPE, leading to the induction of the Toll pathway.

Two-step activation mechanisms are generally observed for zymogens belonging to the chymotrypsin family as they allow spatial (i.e., activation of neutrophil and mast cell serine proteases in the azurophilic granules) or temporal regulation (i.e., activation of plasmin and thrombin during fibrinolysis and coagulation, respectively) to avoid unwanted protein degradation (Caughey, 2016; Collen, 1999; Korkmaz et al., 2008; Wood et al., 2011). In most cases sequential

activation of SPs involves two specific cleavage sites unable to sense a wide range of proteases; however, the long "bait-region" highly sensitive to proteolysis that we described in our study constitutes a novel and original strategy allowing flies to detect exogenous proteases indifferently of their specificities.

Remarkably, the mode of activation of Psh is evocative of the inhibition mechanism of mammalian α2-macroglobulin (α2-M). Indeed, α2-M is a non-specific inhibitor targeting both self- and non-self-proteases and clearing them from tissue fluids (Garcia-Ferrer et al., 2017; Goulas et al., 2017). α2-M contains a 25 amino acid-long bait-region, which is also sensitive to all classes of proteases. Upon cleavage of this "bait-region", α2-M undergoes a structural rearrangement, thus trapping the target proteases (Marrero et al., 2012).

Our work describes a specific detection of infections rather than the recognition of determinants produced by the invading microorganisms; comparable detection mechanisms have been suggested to take place in mammals, where TLRs or Nod-like receptors (NLRs) directly detect virulence factors or endogenous danger signals released by stressed cells and damaged tissues (Creagh and O'Neill, 2006; Shi et al., 2003).

Furthermore, our data revealed the first implication of a circulating cysteine cathepsin in the *Drosophila* immune system. In fact, cysteine cathepsins have long been associated to intracellular functions notably protein turnover inside the endosome/lysosome compartments (Brix et al., 2008), degradation of phagocytized microbes in the phagolysosomes, regulation of MHC class II-dependent antigen presentation (Sadegh-Nasseri and Kim, 2015), or activation of SPs during immune responses: cathepsin C activates neutrophil SPs and granzymes (Hamon et al., 2016) and cathepsin L activates complement protease C3 (Liszewski et al., 2013). At the extracellular level, cysteine cathepsins are correlated with inflammatory-dependent harmful effects, such as degradation of the extracellular matrix or basal membrane components, leading to loss of tissue integrity and microbial dissemination in case of infection. These proteases have been considered as potential therapeutic targets in chronic inflammatory

diseases (Vasiljeva et al., 2007). We believe that the immune function of extracellular cysteine cathepsins in mammals is a topic that deserves further attention.

Animals from fruit flies to humans, as well as plants, are colonized by a range of microorganisms that must be tolerated by the ever evolving and adapting immune response, and understanding how these microorganisms have co-evolved with their hosts is a fascinating topic. Microbial proteases play a key role in the host colonization, in digestive functions and participate in the control of the immune system. Several examples have emerged across species showing that during close host-pathogen co-evolution, immune systems developed the mean to sense danger (Chavarría-Smith et al., 2016; Cheng et al., 2015; LaRock et al., 2016; Turk, 2007; de Zoete et al., 2011). However, due to the high variety of protease enzymatic specificities, such systems are able to detect only a limited number of proteases. Further study of infections, immune defenses and their systemic regulators will continue to open up new routes for translational research and new therapeutic strategies, and result in a more complete understanding of health and disease (Buchon et al., 2014).

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Université de Strasbourg

Ecole Doctorale des Sciences de la Vie et de la Santé STRASBOURG

Najwa ISSA

Détection des protéases microbiennes par la voie immunitaire Toll chez *Drosophila melanogaster*

Résumé en français

Chez la drosophile, l'activation du récepteur Toll menant à une réponse antimicrobienne peut se faire par deux voies différentes. Ces deux voies sont activées soit par des récepteurs dédiés, les Pattern Recognition Receptors (PRRs) reconnaissant des motifs moléculaires microbiens, soit par la coupure d'une molécule circulante appelée Perséphone par des protéases microbiennes extrêmement diverses sécrétées pendant une infection. Cependant, le mécanisme par lequel Perséphone est activée demeurait ambigu.

Nous avons identifié une région unique dans Perséphone fonctionnant comme un appât pour les protéases exogènes indépendamment de leur origine, type ou spécificité. Une coupure dans cette région constitue la première étape d'une activation séquentielle de Perséphone ; elle permet de recruter la cathepsine circulante 26-29-p, qui va générer la forme active de Perséphone.

Ces travaux montrent comment un récepteur de l'immunité innée, Perséphone, peut être activé par un signal de danger, en l'occurrence des enzymes microbiennes, et non par la détection de motifs moléculaires qui peuvent être présents dans la flore microbienne hébergée par les animaux.

Mots-clés : immunité innée, Drosophile, Perséphone, protéases à serine, protéases à cystéine, signaux de danger, protéases exogènes, cathepsine 26-29-p.

Résumé en anglais

In *Drosophila*, the antimicrobial response against infections can be triggered by two different extracellular mechanisms that both lead to the activation of the Toll receptor. These two mechanisms are activated either by the recognition of specific microbial determinants by Pattern Recognition Receptors (PRRs), or by the cleavage of the circulating serine protease Persephone by a wide range of microbial proteases secreted during infections. However, the molecular mechanism underlying Persephone activation remained ambiguous.

We identified a unique region in Persephone pro-domain that functions as a bait for exogenous proteases independently of their origin, type or specificity. Cleavage of Persephone in this bait region constitutes the first step of a sequential activation and licenses the subsequent maturation of Persephone to the endogenous circulating cysteine cathepsin 26-29-p.

Our data establish Persephone itself as an immune receptor able to sense a broad spectrum of microbes through the recognition of danger signals rather than molecular patterns.

Keywords : innate immunity, *Drosophila*, Persephone, serine proteases, cysteine proteases, danger signals, exogenous proteases, cathepsin 26-29-p.